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14. ABSTRACT

Pancreatic cancer is a highly lethal malignancy with an expected 5-year survival of less than 5% for all patients using current therapies. Most of these therapies rely on inducing DNA damage and replication blocks to cause cell death; however the effectiveness of these treatments is often limited to a subset of patients that respond to treatment. Understanding why patients are sensitive or resistant to specific treatments would allow the personalization of therapies that are most effective for a patient while potentially reducing toxicity. The Replication Stress Response (RSR) is a signaling network that recognizes challenges to DNA replication and mobilizes diverse activities to maintain genome integrity. The RSR is critical for the prevention of pancreatic cancer by acting as a barrier against genomic instability and tumorigenesis. We hypothesized that novel RSR genes maintain genome integrity by participating in an ATR-mediated replication stress response and that dysregulation of RSR genes and their cancer barrier function results in the development of pancreatic cancer. Utilizing a custom generated siRNA library targeting genes somatically mutated in pancreatic cancer from the Sanger COSMIC library, we completed a synthetic lethal screen to identify genes which when silenced mediate gemcitabine sensitivity in human pancreatic cancer cells. We further validated positive hits be deconvolution of individual siRNAs and characterized their activities in DNA replication and the DNA damage response. We determined whether these genes were dysregulated and differentially expressed in pancreatic cancer cell lines and patient samples using published data sets.

15. SUBJECT TERMS
Pancreatic cancer, replication stress response, dna damage response, biomarker

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Introduction

Pancreatic cancer is a highly lethal malignancy with an expected 5-year survival of less than 5% for all patients using current therapies. Most of these therapies rely on inducing DNA damage and interfering with DNA replication to cause cell death; however the effectiveness of these treatments is often limited to a subset of patients that respond to treatment. Understanding why patients respond or do not respond to specific treatments would allow the personalization of therapies that are most effective for a patient while potentially reducing toxic side effects. The Replication Stress Response (RSR) is a signaling pathway that recognizes challenges to DNA replication and mobilizes diverse activities to maintain genome integrity. The RSR is critical to prevent pancreatic cancer. In human pre-cancerous lesions, aberrant DNA replication induces activation of the RSR, which maintains genome integrity or causes cell death. Mutations in the RSR promote the survival and proliferation of genetically unstable cells ultimately resulting in cancer. However, the genetic changes that lead to pancreatic cancer can also weaken the ability of cancer cells to respond to treatment by compromising DNA repair pathways. Often the cancer cell will become reliant on backup pathways, which can be targeted to cause cell death through the principle of synthetic lethality. Two genes or pathways are synthetically lethal when inactivation of one is sublethal but inactivation of both causes cell death. We hypothesize that novel RSR genes maintain genome integrity and that deregulation of RSR genes and their cancer barrier function results in the development of pancreatic cancer. However, the genetic changes that drive pancreatic cancer progression also cause susceptibility to synthetic lethal cancer treatments, which can be exploited to personalize therapy. To test this hypothesis, we propose the following specific aims: 1) Identify RSR genes which mediate sensitivity to the first-line chemotherapeutic agent gemcitabine in pancreatic cancer cells. 2) Determine the activities of RSR proteins in DNA replication and DNA damage responses. 3) Evaluate if RSR genes can function as biomarkers for response in pancreatic cancer. Completion of these aims will provide new insights into how the RSR maintains genome integrity, elucidate novel targets for the treatment of pancreatic cancer, and identify subsets of pancreatic cancers that may benefit from gemcitabine chemotherapy.

Keywords

Pancreatic cancer, replication stress response, dna damage response, dna replication, biomarker

Overall Project Summary

We completed a loss of function genetic screen to identify genes, which when silenced cause sensitization or resistance to a low dose of gemcitabine, in human pancreatic cancer cells. We reasoned that genes involved in the RSR would likely be involved in the ATR signaling pathway. We therefore optimized a high-throughput assay using ATR or CHK1 siRNA oligonucleotides as positive controls and ATM or non-targeting (NT) oligonucleotides as negative controls with cell proliferation as a read-out (Fig. 1A-B). The primary screen was completed in MIA PaCa-2 cells, which consistently gave the highest signal to noise ratio among several tested cell types (data not shown). Briefly, cells were reverse transfected with pools of 4 siRNAs targeting a unique sequence of each gene arrayed in a one gene/one well format in 96-well plates. Forty-eight hours after transfection, cells were treated with or without 13 nM gemcitabine for 72 hours prior to assaying for cell proliferation using WST-1 reagent. Each plate contained two positive controls (ATR and CHK1) and several negative controls (NT), and plate-to-plate variability was controlled by normalizing the values on each plate to the average of the negative control values on that plate. We completed three replicas of the primary screen using a

library of 1540 siRNAs, corresponding to four unique siRNA duplexes, targeting each of 385 unique human genes (Fig. 1C) somatically mutated in pancreatic cancer from the Sanger COSMIC database[1] (Task 1-1). Positive hits included a number of genes previously linked to DNA replication and/or DNA damage responses, including *FANCI*, *BRCA2*, *PKP2*, and *CTBP2*, demonstrating that our screen can yield genes involved in the RSR.

We performed bioinformatic analyses of our positive hits including cross-referencing with published putative ATM/ATR substrates[2] and published DNA damage sensitivity screens and putative ATM/ATR substrates[3-6] (Task 3-1). Using these criteria, we selected 20 genes for further analysis in a secondary screen by deconvoluting with individual siRNAs (Task 1-2). Ten of our genes had at least 2 siRNAs causing sensitivity phenotypes, including chromodomain helicase DNA binding protein 7 (CHD7), which we focused on for further analysis. Four of four siRNAs targeting CHD7 caused gemcitabine sensitization (Fig. 2A). Western blot analysis confirmed decreased levels of CHD7 following siRNA knockdown as well as specificity of the anti-CHD7 antibody used for immunohistochemistry (IHC) analysis (Fig. 2B) (Task 3-3). A similar gemcitabine sensitization after CHD7 silencing was observed using a range of gemcitabine concentrations and in BxPC-3 and HPAC pancreatic cancer cells, suggesting that the phenotype is not cell-type specific (data not shown) (Task 1-3). We also determined the gemcitabine sensitivity of CHD7 depleted cells using a colony formation assay. MIA PaCa-2 cells silenced for CHD7 demonstrated a significantly reduced percentage of surviving colonies following a 24 hour pulse of gemcitabine in a dose-dependent manner compared to a NT control (Fig. 2C), confirming the gemcitabine sensitization of CHD7 depleted cells observed with WST-1 reagent. Consistent with these findings, MIA PaCa-2 and BxPC3 pancreatic cancer cells, which express lower levels of CHD7 than HPAC, CAPAN-1, and AsPC-1 pancreatic cancer cells, demonstrated increased gemcitabine sensitivity (Fig. 2D-E), suggesting that CHD7 expression may predict response to gemcitabine in pancreatic cancer cells (Task 3-5, 3-6).

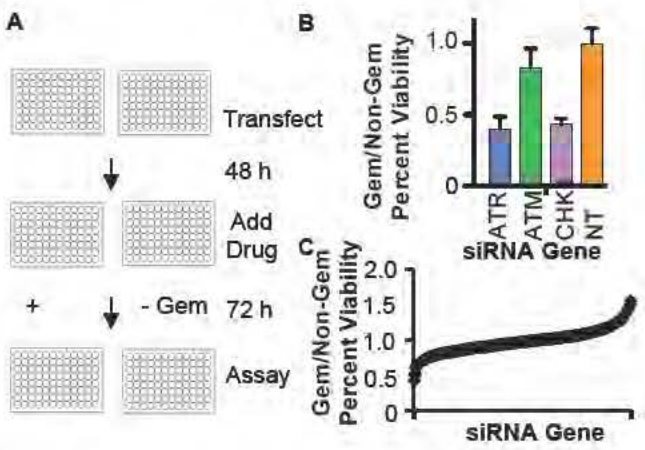

Fig. 1. Gemcitabine sensitivity screen. A. Primary screen flow diagram. MIA PaCa-2 cells were transfected with siRNA (pools of 4 siRNAs per gene) in a one gene/one well format in 96-well plates. 48 hours after transfection, cells were treated with or without 13 nM gemcitabine for 72 hours prior to assaying for cell proliferation using WST-1 reagent. In each plate, non-targeting (NT) targeting, ATR, and CHK1 siRNA were used as controls. Plate-to-plate variability was controlled by normalizing the values on that plate to the average of the negative controls on that plate. **B.** Optimization of cell viability assay using ATR, ATM, CHK1, or NT siRNA. The viability ratio of gemcitabine treated to untreated is shown. Mean and standard deviation from three replicas is shown. **C.** Results of primary screen. The primary screen was completed in triplicate. The viability ratio of treated versus untreated cells is shown.

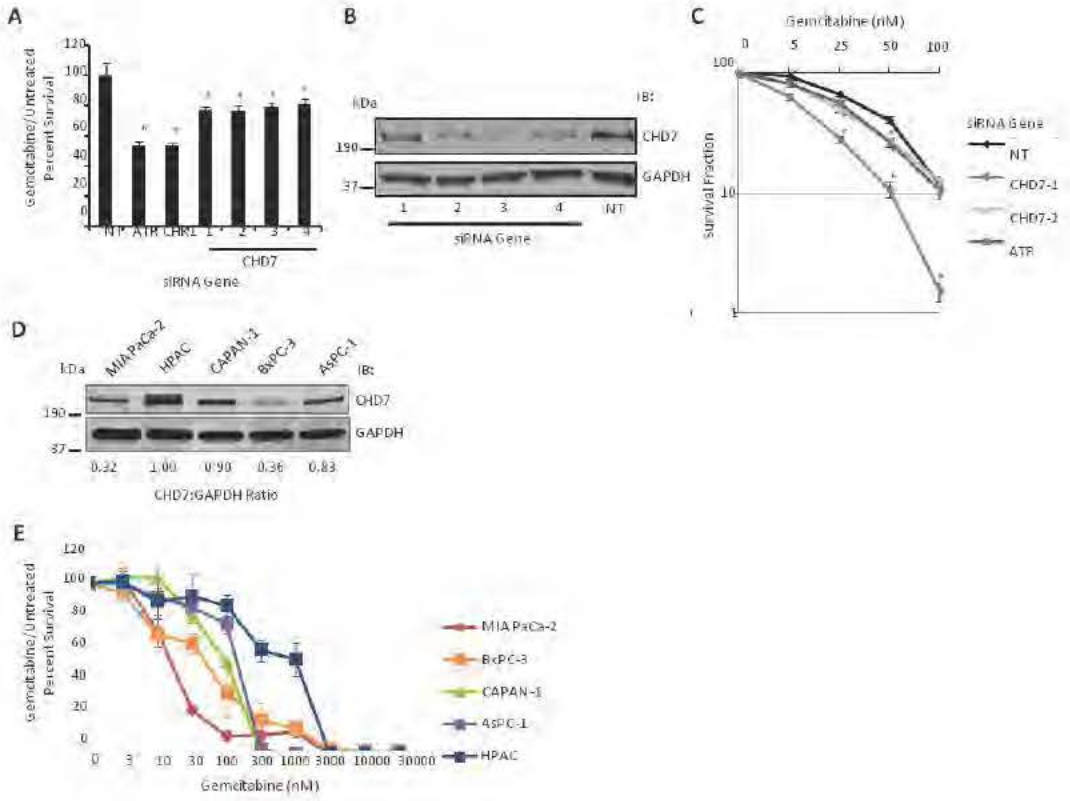

Fig 2. CHD7 knockdown causes gemcitabine sensitization. A. Four siRNAs targeting CHD7 caused gemcitabine sensitization in MIA PaCa-2 cells. Treated versus untreated percent viability was calculated and the mean and standard deviation from three replicas is shown. * indicates $p < 0.05$. **B.** Western blot analysis demonstrating efficiency of CHD7 knockdown with indicated siRNAs. **C.** Clonogenic assay demonstrating gemcitabine sensitization with CHD7 silencing. MIA PaCa-2 cells transfected with siRNA against CHD7, ATR, or NT were seeded for colony formation, treated with indicated concentrations of gemcitabine for 24 hours, and assayed for surviving colonies 8-12 days later. Percent survival of colonies from treated versus untreated cells is indicated. Mean and standard deviation from three replicas are shown. * indicates $p < 0.05$. **D.** Western blot analysis of cell lysate from MIA PaCa-2, HPAC, CAPAN-1, BxPC-3, and AsPC-1 cells with the indicated ant bodies. The CHD7:GAPDH ratio of representative blot from three independent experiments is shown. **E.** Gemcitabine sensitivity of MIA PaCa-2, HPAC, CAPAN-1, BxPC-3, and AsPC-1 cells following treatment with indicated concentrations of gemcitabine for 72 hours is shown. * indicates $p < 0.05$.

The gemcitabine hypersensitivity of CHD7 depleted cells suggests that CHD7 may function in the RSR. CHD7 silencing significantly increased the percentage of cells staining with γH2AX, a marker for DNA damage, following treatment with gemcitabine (Fig. 3A), suggesting that CHD7 silencing potentiates gemcitabine-induced DNA damage (Task 2). However, no significant difference in repair kinetics was observed between cells silenced with CHD7 compared with a NT siRNA (Fig. 3A) (Task 2). To determine if CHD7 is involved in cell cycle progression, we examined cells for incorporation of BrdU using flow cytometry for DNA content (Task 2-1). CHD7 silenced cells showed a decreased percentage of cells in S-phase and an increased percentage of cells in G2/M phase compared with control cells (Fig. 3B). To determine if CHD7 functions in ATR-dependent signaling in response to gemcitabine treatment, we examined cells for the phosphorylation of CHK1 Ser317. CHD7 silencing significantly reduced CHK1 Ser317 phosphorylation but not total CHK1 protein levels in response to gemcitabine treatment (Fig. 3C), suggesting that CHD7 functions in controlling ATR dependent

phosphorylation of CHK1 in response to gemcitabine treatment (Task 2-4). To determine if CHD7 functions in DNA damage responses, we examined cells for S-phase and G2/M checkpoint induction following ionizing radiation but found no evidence that CHD7 is involved in the S-phase or G2/M checkpoint (data not shown) (Task 2-2, Task 2-3). We also performed mass spectrometry of purified HA-CHD7 expressed in cells (Task 2-5, Task 2-8) to examine for potential interacting partners that may function in the RSR but found no evidence that CHD7 interacts in a complex with known RSR proteins (data not shown)

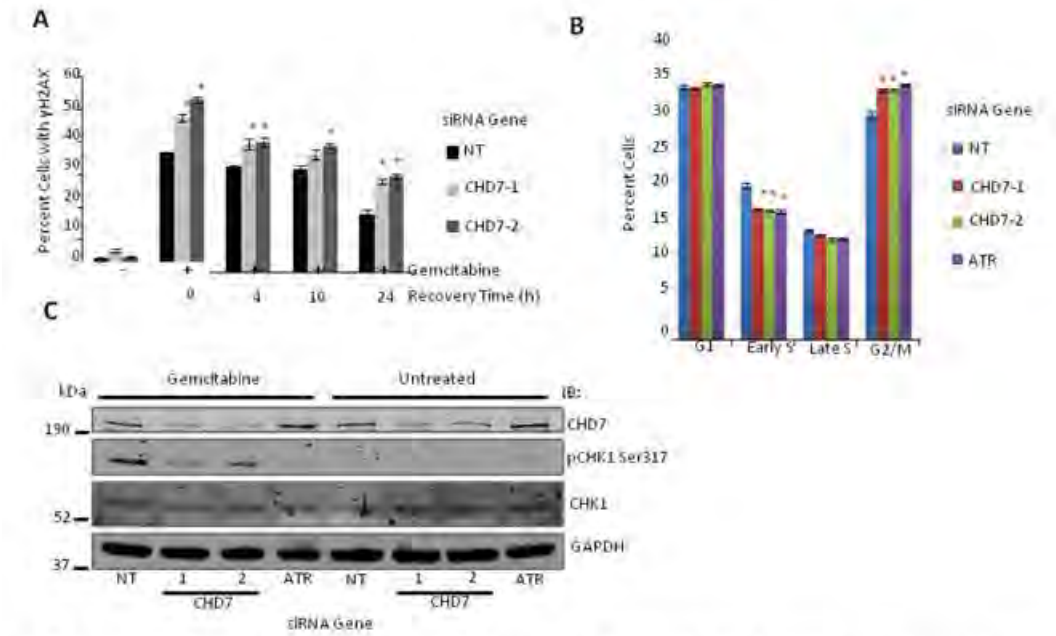

Fig 3. CHD7 is a DNA damage response protein. A. MIA PaCa-2 cells were treated with or without gemcitabine for 20 hours, washed, released for the indicated time points, and processed for γH2AX staining by indirect immunofluorescence. The percentage (mean and standard deviation) of γH2AX positive cells from three replicas is shown. * indicates $p < 0.05$ **B.** MIA PaCa-2 cells were transfected with NT, CHD7, or ATR siRNA, labeled with BrdU, fixed, and processed for BrdU incorporation and DNA content by flow cytometry. Mean and standard deviation of gated cells from three replicas is shown. **C.** Western blot analysis of cell lysate from MIA PaCa-2 cells treated with or without gemcitabine for 6 hours and probed with anti-CHD7, pCHK-1 Ser317, CHK1, and GAPDH antibodies.

Key Research Accomplishments

1. Identified novel RSR genes that are critical for determining gemcitabine sensitivity in pancreatic cancer.
2. Characterized functions of novel RSR proteins, including showing that CHD7 functions in the ATR signaling pathway
3. Determined that expression of CHD7 can function as a biomarker for gemcitabine sensitivity in cells

Conclusion

We identified a number of novel RSR genes that are critical for determining gemcitabine sensitivity in cells and tumors, including CHD7. We determined that CHD7 functions as a novel replication stress response protein in the ATR signaling pathway. We also found that CHD7

expression is associated with gemcitabine sensitivity in pancreatic cancer cells. We are now working to determine whether expression of CHD7 and other genes identified in our screen can be utilized as prognostic and predictive biomarkers to personalize treatment for patients with pancreatic cancer.

"Publications, Abstracts, Presentations"

Manuscripts:

1. Lay Press:
   Nothing to report

2. Peer-Reviewed Scientific Journals:

   Hall, W.A., Petrova, A.V., Colbert, L.E., Hardy, C.W., Fisher, S.B., Saka, B., Shelton, J.W., Warren, M.D., Pantazides, B.G., Gandhi, K., Kowalski, J., Kooby, D.A., El-Rayes, B.F., Staley, C.A., Adsay, N.V., Curran, W.J., Landry, J.C., Maithel, S.K., **Yu, D.S.** 2013. Low CHD5 expression activates the DNA damage response and predicts poor outcome in patients undergoing adjuvant therapy for resected pancreatic cancer. **Oncogene**, DOI:10.1038/onc.2013.488.

   Colbert, L.E., Petrova, A.V., Fisher, S.B., Pantazides, B.G., Madden, M.Z., Hardy, C.W., Warren, M.D., Pan, Y., Nagaraju, G.P., Liu, E.A., Saka, B., Hall, W.A., Shelton, J.W., Gandhi, K., Pauly, R., Kowalski, J., Kooby, D.A., El-Rayes, B.F., Staley, C.A., Adsay, N.V., Curran, W.J., Landry, J.C., Maithel, S.K., **Yu, D.S.** 2014. CHD7 expression predicts survival outcomes in patients with resected pancreatic cancer. **Cancer Research**, 74(10), 2677-87. PMCID: PMC4025946.

3. Invited Articles:
   Nothing to report

4. Abstracts:

   Landry, J.C., Pantazides, B.G., Park, W., Shelton, J.W., Maithel, S.K., El-Rayes, B., Yu, D.S. 2012. A synthetic lethal screen identifies genetic determinants for gemcitabine sensitivity in pancreatic cancer. American Association for Cancer Research (AACR) Annual Meeting, Chicago, IL. Poster presentation.

   Hardy, C.W., Pantazides, B.G., Gandhi, K., Landry, J., Shelton, J., Maithel, S., El-Rayes B., Kowalski, J., Yu, D.S. 2012. A loss of function genetic screen identifies determinants of gemcitabine sensitivity in pancreatic cancer. AACR/Pancreatic Cancer: Progress and Challenges Meeting, Lake Tahoe, NV. Poster presentation.

   Pantazides, B.G., Hardy, C.W., Gandhi, K., Landry, J., Shelton, J., Maithel, S., El-Rayes B., Kowalski, J., Yu, D.S. 2012. A synthetic lethal screen identifies genes that mediate gemcitabine resistance in pancreatic cancer. Radiation Research Society Annual Meeting, San Juan, Puerto Rico. Poster presentation.

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gemcitabine resistance in pancreatic cancer. American Society for Therapeutic Radiology and Oncology (ASTRO) 54th Annual Meeting, Boston, MA. Oral presentation.

Warren, M.D., Hardy, C.W., Pantazides, B.G., Landry, J.C., Shelton, J.W., Maithel, S.K., El-Rayes, B., Kowalski, J, Yu, D.S. 2013. A synthetic lethal screen identifies determinants of gemcitabine sensitivity in pancreatic cancer. AACR Annual Meeting, Washington, DC. Poster presentation.

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Presentations:

Landry, J.C., Pantazides, B.G., Park, W., Shelton, J.W., Maithel, S.K., El-Rayes, B., Yu, D.S. 2012. A synthetic lethal screen identifies genetic determinants for gemcitabine sensitivity in pancreatic cancer. American Association for Cancer Research (AACR) Annual Meeting, Chicago, IL. Poster presentation.

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"Inventions, Patents, Licenses"

Nothing to report

Reportable Outcomes

Nothing to report

Other Achievements

Nothing to report

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Appendices

Cancer Research

CHD7 Expression Predicts Survival Outcomes in Patients with Resected Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease with poor outcomes with current therapies. Gemcitabine is the primary adjuvant drug used clinically, but its effectiveness is limited. In this study, our objective was to use a rationale driven approach to identify novel biomarkers for outcome in patients with early stage resected PDAC treated with adjuvant gemcitabine. Using a synthetic lethal screen in human PDAC cells, we identified 93 genes, including 55 genes linked to DNA damage responses (DDR), that demonstrated gemcitabine sensitization when silenced, including CHD7, which functions in chromatin remodeling. CHD7 depletion sensitized PDAC cells to gemcitabine and delayed their growth in tumor xenografts. Moreover, CHD7 silencing impaired ATR dependent phosphorylation of CHK1 and increased DNA damage induced by gemcitabine. CHD7 was dysregulated, ranking above the 90th percentile in differential expression in a panel of PDAC clinical specimens, highlighting its potential as a biomarker. Immunohistochemical analysis of specimens from 59 patients with resected PDAC receiving adjuvant gemcitabine revealed that low CHD7 expression was associated with increased recurrence free survival (RFS) and overall survival (OS), in univariate and multivariate analyses. Notably, CHD7 expression was not associated with RFS or OS for patients not receiving gemcitabine. Thus, low CHD7 expression was correlated selectively with gemcitabine sensitivity in this patient population. These results supported our rationale driven strategy to exploit dysregulated DDR pathways in PDAC to identify genetic determinants of gemcitabine sensitivity, identifying CHD7 as a novel biomarker candidate to evaluate further for individualizing PDAC treatment. *Cancer Res; 74(10); 2677 87. ©2014 AACR.*

Introduction

Pancreatic adenocarcinoma has a poor prognosis with a 5 year overall survival (OS) rate around 5% (1). Patients with early stage pancreatic adenocarcinoma who undergo resection demonstrate the best prognosis, particularly when resection is followed by adjuvant chemotherapy with or without radiother apy (2, 3). Still, recurrence is common and OS remains poor even for patients who undergo complete resection and adju vant therapy. Recent developments have suggested that pan creatic adenocarcinoma is a genetically heterogeneous disease (4) and, as such, patients may benefit from the identification of predictive biomarkers for responsiveness to adjuvant therapy.

Gemcitabine is the primary chemotherapeutic agent used to treat patients with pancreatic adenocarcinoma in the adjuvant setting (2, 5). The cytotoxic effects of gemcitabine are mediated in part through incorporation into DNA as a terminal nucle oside analog and in part through inhibition of ribonucleotide reductase, which depletes nucleotides required for DNA syn thesis. However, the efficacy of gemcitabine for pancreatic adenocarcinoma is limited. A better understanding of which patients are likely to respond to gemcitabine treatment would facilitate personalization of therapy and optimize the clinical benefit to toxicity ratio associated with adjuvant therapy.

The DNA damage response (DDR) pathway is critical for the maintenance of genome integrity and serves as a cancer barrier by mobilizing DNA repair, cell cycle arrest, and/or apoptosis (6, 7). In human precancerous lesions, aberrant DNA replica tion induces DDR activation, which constrains tumor devel opment. Thus, the DDR acts as a barrier against genomic instability and cancer development. Tumor cells may in turn develop mutations or epigenetic silencing of protective DDR genes, leading to the proliferation of genetically unstable cells and ultimately resulting in cancer. Indeed, a large number of DDR genes are somatically mutated in pancreatic

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adenocarcinoma, including *ATM*, *BRCA2*, *CDKN2A*, *FANCI*, *HELB*, and *RAD9* (8). These genetic changes in the DDR pathway can lead to pancreatic adenocarcinoma and can also weaken the ability of cancer cells to respond to treatment by decreasing activity in DNA repair pathways. Often, the cancer cell will become reliant on backup pathways that can be targeted to cause cell death through the principal of synthetic lethality (inactivation of one gene or pathway is sublethal but inactivation of both causes cell death). As such, determining genetic alterations and cancer treatments that are syntheti cally lethal may lead to the identification of novel druggable targets as adjuncts to gemcitabine treatment or novel biomar kers to predict response to gemcitabine therapy. Using this rationale, we sought to exploit dysregulated DDR pathways in pancreatic adenocarcinoma by identifying genetic determi nants that are synthetically lethal with gemcitabine treatment and evaluating their clinical relevance as biomarkers for out come in patients with early stage resected pancreatic adeno carcinoma treated with adjuvant gemcitabine.

Chromodomain helicase DNA binding protein 7 (*CHD7*), is a member of a family of chromodomain enzymes that belong to the ATP dependent chromatin remodeling protein SNF2 super family. Mutations in *CHD7* lead to congenital CHARGE syn drome, named for its characteristic traits: *c*oloboma of the eye, *h*eart defects, *a*tresia of the nasal choanae, *r*etardation of growth and/or development, *g*enital and/or urinary abnormalities, and *e*ar abnormalities and deafness (9), and Kallman Syndrome, a genetic disorder marked by hypogonadotropic hypogonadism and anosmia (10). *CHD7* is also dysregulated in 13% to 35% of cases of pancreatic adenocarcinoma, with aberrant expression, copy number variation, and somatic mutations (see Supple mentary Table S3; refs. 11 13). *CHD7* helps to regulate neural crest gene expression (14), regulates ribosomal RNA biogenesis (15), and interacts with SOX2 to regulate gene expression (16). *CHD7* is also a putative substrate of the ATM/ATR checkpoint kinases, suggesting that it may play a role in the DDR (17, 18). The clinical significance of *CHD7* expression in pancreatic adenocarcinoma has not previously been reported.

The purpose of this analysis was to use a rationale driven approach to identify novel biomarkers for outcome in patients with early stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine (Fig. 1A). We initially completed a synthetic lethal siRNA screen to identify genetic determinants of gemcitabine sensitivity in human pancreatic cancer cells and identified the top 15% of these genes for further analysis. Genes validated by a secondary screen and/or linked to the DDR were then analyzed for dysregulation and differential expression in pancreatic adenocarcinoma by mining published data sets to determine their potential as biomarkers. Finally, we correlated CHD7 gene expression characterized by immunohistochemistry (IHC) with clinical outcome in patients with early stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine.

Materials and Methods

Cell culture, siRNA, and transfection

MIA PaCa 2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% FBS (Gibco) and 2.5% horse serum (Gibco). HPAC cells were grown in 1:1 DMEM:Hams F12 supplemented (Gibco) with 40 ng/mL hydro cortisone, 10 ng/mL EGF, and 5% FBS. BxPC 3 and AsPC 1 cells were grown in RPMI 1640 supplemented with 10% FBS, and CAPAN 1 cells were grown in Iscove's Modified Dulbecco's Medium supplemented with 20% FBS. Cell lines were grown in a humidified incubator at 37°C with 5% carbon dioxide.

Transfections were done using the HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocol. Primary and secondary screen siRNAs were purchased from Thermo Scientific. siRNA sequences are listed below.

NT: (ATGAACGTGAATTGCTCAATT)
ATR: (CCUCCGUGAUGUUGCUUGA)
ATRIP: (GGTCCACAGATTATTAGA)
CHK1: (CTGAAGAAGCAGTCGCAGT)
CHD7 1: (UAACGUACCUAACCUAUUA)
CHD7 2: (CGACAAGGCUAGUUUGAAA)
CHD7 3: (GGGAAGCUAUUAUAUCUGA)
CHD7 4: (GUAGAUAACCAAGAACUAA)

TRC lentiviral short hairpin RNA (shRNA) was purchased from Thermo Scientific: shControl (RHS4080), shCHD7 1 (1RHS3979 201747986), shCHD7 2 (1RHS3979 201747990).

Gemcitabine sensitivity screen

MIA PaCa 2 cells were transfected in 96 well plates using HiPerFect Transfection Reagent (Qiagen) with 25 nmol/L siRNA from a custom siGENOME siRNA library (Thermo Scientific) of 4,024 siRNAs corresponding to 1,006 unique human nuclear enzyme genes (pools of four siRNAs targeting a unique sequence of each gene) using a one gene per well format. Twenty four hours later, plates were split 1:4, and then treated following another 24 hours with or without 13 nmol/L gemcitabine (Hospira, Inc.) for 72 hours before assaying for cell proliferation using WST 1 reagent (Roche Diagnostics). Each plate contained two positive controls (ATR and CHK1) and several negative controls (NT), and plate to plate variability was controlled by normalizing the values on each plate to the average of the negative control values on that plate. A ratio of gemcitabine treated/untreated viability was calculated and normalized to that of nontargeting siRNA. Principal compo nents analysis (PCA) was used to account for possible vari ability between the cell viability of the three replicates for each gene. These genes were then sorted by increasing average cell viability via PCA, and the top 15% of genes were categorized as possible "hits."

Secondary validation screen

MIA Pa Ca 2, BxPC3, or HPAC cells were transfected in 96 well plates with 25 nmol/L siRNA, split 1:4 24 hours later, and then treated following another 24 hours with or without gemcitabine at IC_5, IC_{25}, or IC_{50} for 72 hours before assaying for cell proliferation using WST 1 reagent. A ratio of gem citabine treated/untreated viability was calculated and normalized to that of nontargeting siRNA. MIA PaCa 2, HPAC, CAPAN 1, BxPC3, and AsPC 1 cells were treated

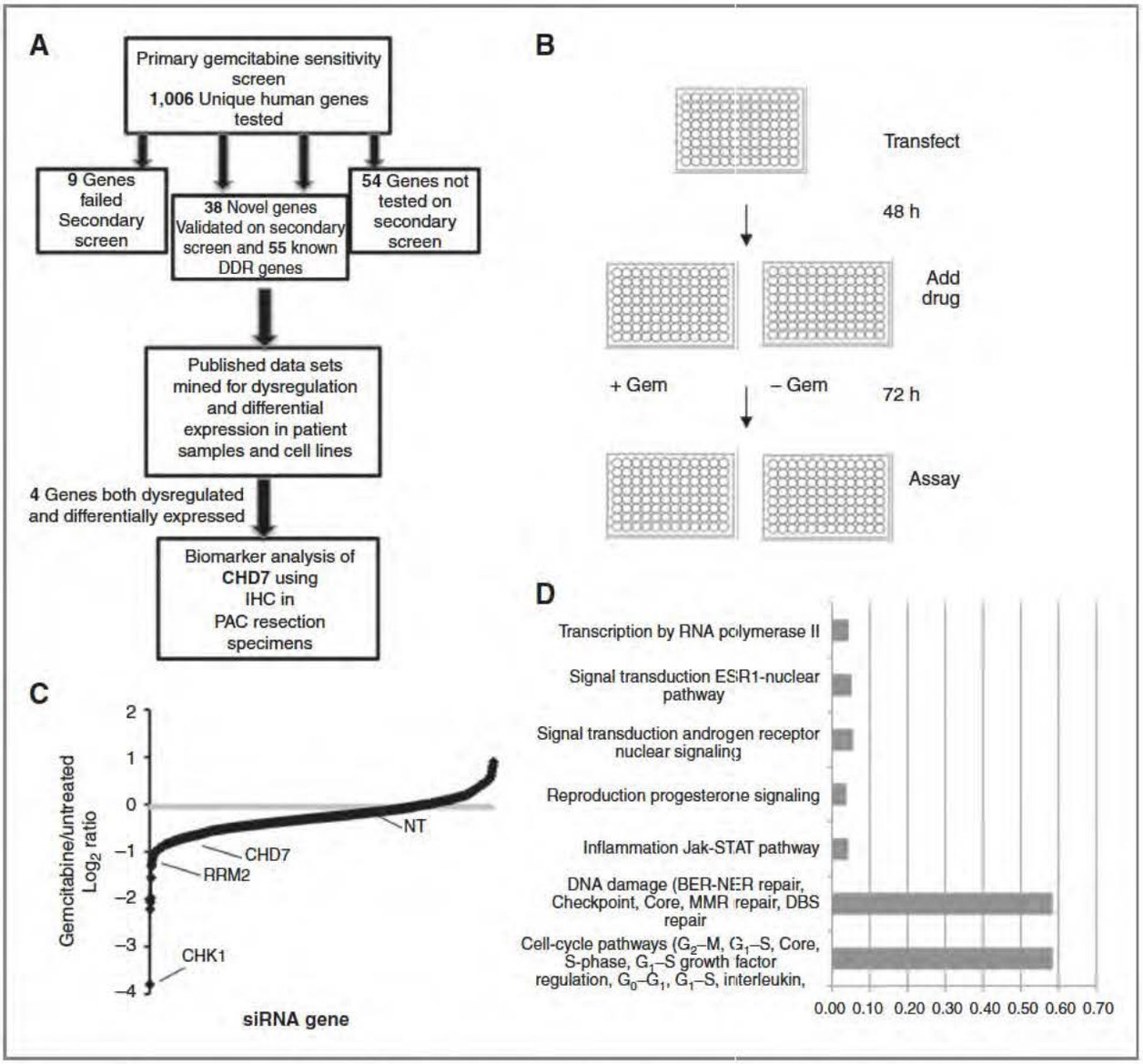

Figure 1. Primary gemcitabine sensitivity screen. A, flow diagram of approach for identifying novel biomarkers for outcome in patients with early stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine. B, diagram of primary screen as described in text. C, results of primary screen. The $\log_2$ ratio of treated versus untreated cell viability relative to the nontargeting (NT) siRNA for each gene is shown. Mean from three replicas of primary screen is shown. D, proportion of top 15% of gemcitabine sensitivity genes with statistically significant involvement in known pathways. The top 18 pathways identified on network analysis via MetaCore ExPlain Process Network Analysis can be consolidated into the listed categories. Fifty five percent of all identified genes were involved in DDR pathways.

with the indicated concentrations of gemcitabine for 72 hours before assaying for cell proliferation using the WST 1 reagent.

Colony formation assay

Cells were transfected with 25 nmol/L siRNA. Following a 24 hour knockdown, 500 cells were seeded into 6 well plates in triplicate. Cells were allowed to culture overnight and were then treated for 24 hours with increasing concentrations of gemcitabine. Following the gemcitabine incubation, the plates were washed with PBS and fresh media were added for 8 to 12 days before staining colonies with a 0.5% crystal violet (Ampresco) solution.

Western blot analysis

MIA PaCa 2 cells transfected with siRNA for 48 hours or MIA PaCa 2, HPAC, CAPAN 1, BxPC3, and AsPC 1 cells were harvested with NP40 buffer containing 200 mmol/L NaCl, 1% NP40, 50 mmol/L Tris HCl (pH 8.0), and supplemented with fresh protease inhibitors. Samples were loaded into a SDS PAGE gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and subsequently probed with an anti CHD7

antibody (NBP1 77393; Novus Biologicals) and anti GAPDH antibody (GeneTex; GTX627408) followed by LI COR IRDye secondary antibodies. Detection was performed using the Odyssey system (LI COR Biosciences).

To analyze phosphorylation of CHK1, MIA PaCa 2 cells were transfected with 25 nmol/L siRNA for 48 hours and treated with 1 mmol/L gemcitabine for 6 hours. Cells then were harvested, washed with PBS, and lysed in cold RIPA buffer (25 mmol/L Tris HCl pH 7.6, 150 mmol/L NaCl, 1% NP40, 1% sodium deoxycholate, and 0.1% SDS) with protease and phosphatase inhibitors for 30 minutes. Lysates were clarified by centrifugation at 16 $\times g$ for 10 minutes and 150 μg of protein for each sample were used for Western blot analysis. Primary antibody pCHK1 S317 (Cell Signaling Technology; #2344) and CHK1 (Santa Cruz Biotechnology; sc 8408) were used for detection of phosphorylated and total CHK1, respectively.

In vivo tumor growth inhibition assay

Male nude mice were maintained in a pathogen free environment, and all *in vivo* procedures were approved by the Emory University, Institutional Animal Care and Use Committee. Stable MIA PaCa 2 shCHD7 2 and MIA PaCa 2 shControl cells (1.5×10^6/0.1 mL of 20% Matrigel gel in serum free media) were injected subcutaneously into the flank of 5 week old mice. Mice bearing established tumors (100 to 125 mm^3) were randomized into treatment groups of four. Animals were treated on days 0, 7, and 14 via the tail vein with either vehicle or 100 mg/kg gemcitabine. Tumor growth inhibition was determined as described previously (19).

γH2AX DNA damage assay

MIA PaCa 2 cells were treated with or without 13 nmol/L gemcitabine for 20 hours, washed, released for the indicated time points, and processed for γH2AX staining by indirect immunofluorescence. Cells were washed with 1× PBS, fixed with 2% paraformaldehyde for 10 minutes at room temperature, permeabilized with 0.5% Triton X 100 (Fisher Scientific), and blocked in a 5% bovine serum albumin solution (Sigma). Cells were then immunostained with anti phospho histone H2AX (Ser139) antibody (Millipore; 05 636) and anti mouse secondary antibody with AlexaFluor 488 (Invitrogen; A21206). After incubation, cells were mounted onto slides with a mounting media containing 4′, 6 diamidino 2 phenylindole (DAPI) and dried. Analysis was performed using a Zeiss Observer Z1 microscope with Axiovision Rel 4.8 software using the 63× oil objective. Foci quantitation was conducted by counting 250 healthy cells and scoring cells with 10 or more foci as positive. Experiments were done in triplicate.

Cell cycle analysis

MIA PaCa 2 cells were transfected with 25 nmol/L siRNA for 48 hours and treated with or without gemcitabine (13 nmol/L) for 24 hours. After fixing with ice cold 70% ethanol, cells were washed in PBS, and propidium iodide (PI; 25 μg/mL; Sigma) and RNase A (10 μg/mL; Qiagen) were added to determine DNA content. Cells were analyzed on a FACSCanto II (BD Biosciences) and FlowJo software.

Biomarker selection

Gemcitabine sensitivity genes validated on secondary screen or known DDR genes were analyzed for evidence of dysregulation by identifying genes overexpressed in The Compendium of Potential Biomarkers of Pancreatic Cancer (20) or somatically mutated in the pancreatic Catalogue for Somatic Mutations in Cancer (COSMIC) database (11). For determination of differential expression, we extracted expression data from the two Gene Expression Omnibus (GEO) submissions based on the Affymetrix U133 Plus 2.0 platform: (i) GSE12654, a 22 pancreatic cancer cell line study (19); and (ii) GSE16515, 20 pancreatic patient tumors (21, 22). Within each study, after processing and normalization, we performed a genomewide filter to identify genes with "large" expression differences among tumors, and separately, among cell lines, using a variance approach. We define "differential expression" as genes whose expression variability is "large" relative to all other genes on the array, in which "large" is defined according to whether expression variability associated with a gene was greater than the 90th percentile from all genes. We then compared this list of genes with the lists in Supplementary Tables S2 and S4. *CHD7* was chosen as a potential biomarker based on evidence of both dysregulation and differential expression.

IHC patient selection

Patients were selected for this analysis from a prospectively maintained database of patients who underwent resection for early stage pancreatic adenocarcinoma between January 2000 and October 2008; data for these patients have been included in other cohorts previously reported (23 26). These 59 patients received adjuvant chemotherapy with or without adjuvant radiation. The gemcitabine patient population was composed of 42 of these patients who received gemcitabine as a component of the adjuvant chemotherapy regimen. An additional 17 patients received adjuvant chemotherapy with agents other than gemcitabine. OS was calculated from date of surgery to patient death. Recurrence free survival (RFS) was measured on the basis of surveillance imaging obtained at regular intervals after resection. Patient demographics, pathologic characteristics, and treatment characteristics were originally collected from pathologic record and chart review. Permission was obtained from the Emory Institutional Review Board 00048816, and patient confidentiality was maintained according to the Health Insurance and Patient Accessibility Act of 1996.

Immunohistochemical analysis

An experienced pathologist identified representative sections of tumor and normal tissue from formalin fixed paraffin embedded slides. The tissue was stained using an anti CHD7 mouse monoclonal antibody (NBP1 77393; Novus Biologicals) at a concentration of 1:200. Specificity of the anti CHD7 antibody was validated by Western blot analysis following siRNA silencing (Fig. 2B). An expression score was calculated using a previously defined scoring system (23, 27). Overall score was dichotomized into low (<3.1) and high (>3.1) expression groups for this analysis (Supplementary Fig. S1).

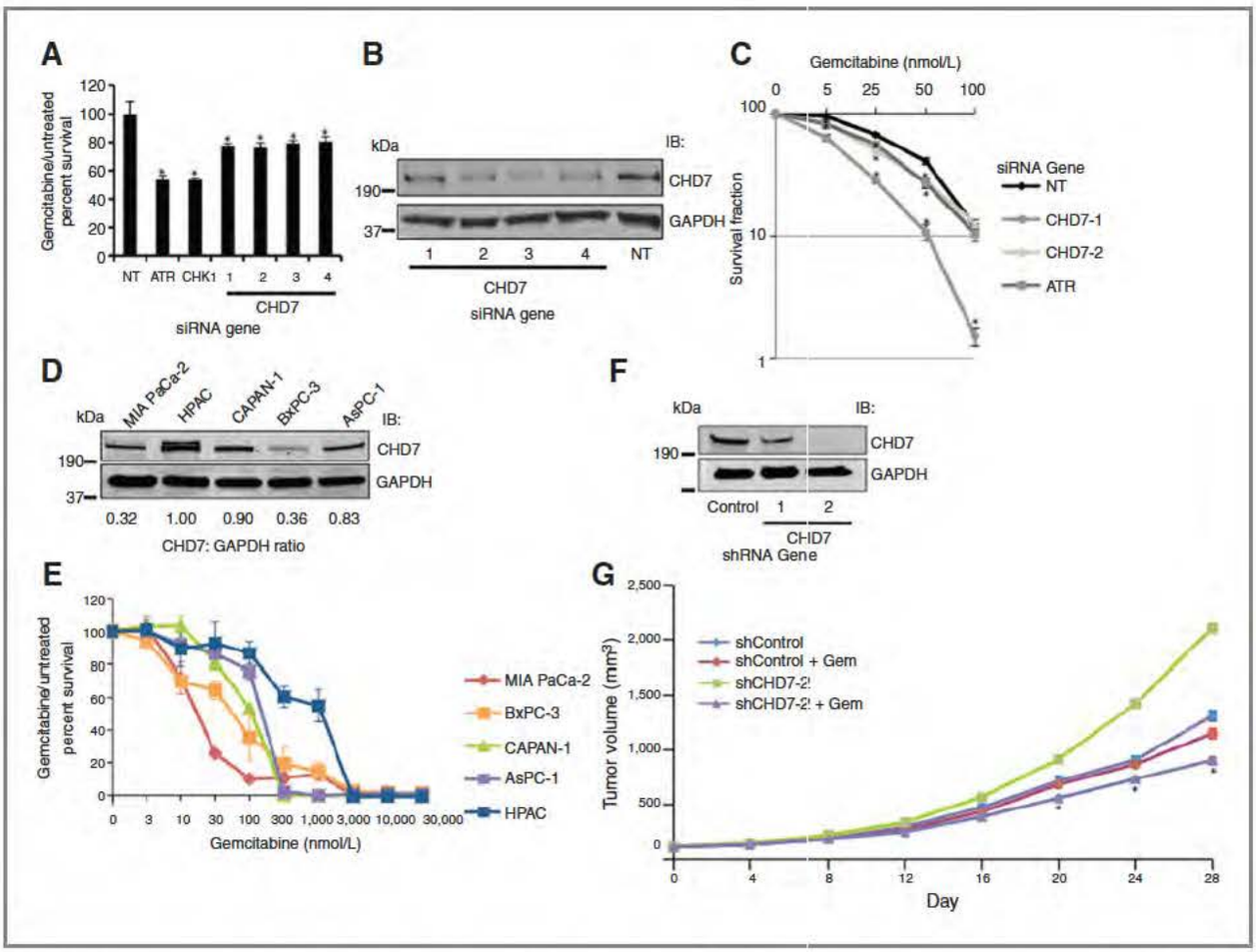

Figure 2. CHD7 knockdown causes gemcitabine sensitization. A, four siRNAs targeting CHD7 caused gemcitabine sensitization in MIA PaCa 2 cells. Treated versus untreated percent viability was calculated and the mean and SD from three replicas is shown; *, $P < 0.05$. B, Western blot analysis demonstrating efficiency of CHD7 knockdown with indicated siRNAs. C, clonogenic assay demonstrating gemcitabine sensitization with CHD7 silencing. MIA PaCa 2 cells transfected with siRNA against CHD7, ATR, or NT were seeded for colony formation, treated with indicated concentrations of gemcitabine for 24 hours, and assayed for surviving colonies 8 to 12 days later. Percent survival of colonies from treated versus untreated cells is indicated. Mean and SD from three replicas are shown; *, $P < 0.05$. D, Western blot analysis of cell lysate from MIA PaCa 2, HPAC, CAPAN 1, BxPC 3, and AsPC 1 cells with the indicated antibodies. The CHD7:GAPDH ratio of representative blot from three independent experiments is shown. E, gemcitabine sensitivity of MIA PaCa 2, HPAC, CAPAN 1, BxPC 3, and AsPC 1 cells following treatment with indicated concentrations of gemcitabine for 72 hours is shown. F, Western blot analysis demonstrating efficiency of CHD7 knockdown with indicated shRNAs in MIA PaCa 2 cells. G, athymic nude mice with shCHD7 and shControl MIA PaCa 2 tumor xenografts were treated with or without gemcitabine (100 mg/kg) on days 0, 7, and 14, and tumor growth was measured every 4 days. Mean and SEM from six tumors are shown; *, $P < 0.05$.

Statistical analysis

Descriptive statistics were generated for patient characteristics, tumor characteristics, and treatment characteristics. Similar statistical analyses were performed for patients receiving adjuvant therapy, patients receiving gemcitabine based therapy, and patients receiving non gemcitabine based therapy. Kaplan Meier log rank survival analysis was performed to determine prognostic factors for RFS and OS. Univariate and multivariate Cox regression analyses were performed for all patients to examine the correlation of CHD7 expression level on both RFS and OS. Factors examined on univariate analysis included age, sex, ethnicity, receipt of adjuvant and neoadjuvant therapy, tumor size, margin status, grade, nodal status, perineural invasion, lymphovascular invasion, receipt of radiotherapy, CA19 9 levels, and type of adjuvant chemotherapy. Clinically relevant covariates significant to a level of $P < 0.2$ on univariate analysis for either RFS or OS were included in the multivariate model; these included tumor size, margin status, nodal status, perineural invasion, lymphovascular invasion, and tumor grade. Data were analyzed using the Statistical Package for the Social Sciences 19.0 software for Windows (IBM).

Results

Gemcitabine sensitivity screen

To identify genetic determinants of gemcitabine sensitivity, we completed a siRNA screen to identify genes that when silenced cause either sensitization or resistance to a low dose of

gemcitabine in human pancreatic cancer cells. Because gemcitabine induces DNA damage and replication stress, we reasoned that gemcitabine sensitivity genes would likely be involved in the DDR. We, therefore, optimized a high throughput assay using ATR and CHK1 siRNA as positive controls and a nontargeting siRNA as a negative control with cell proliferation as a read out (Fig. 1B). The primary screen was completed in MIA PaCa 2 cells, which consistently gave the highest signal to noise ratio among several tested cell types (Supplementary Fig. S3A and data not shown). Briefly, cells were transfected with pools of four siRNAs targeting a unique sequence of each gene arrayed in a one gene per one well format in 96 well plates. Forty eight hours after transfection, cells were treated with or without 13 nmol/L gemcitabine (equivalent to IC_{25} under these conditions, see Fig. 2E) for 72 hours before assaying for cell proliferation using WST 1 reagent. Each plate contained two positive controls (ATR and CHK1) and several negative controls (NT), and plate to plate variability was controlled by normalizing the values on each plate to the average of the negative control values on that plate. We completed three replicas of the primary screen using a library of 4,024 siRNAs, corresponding to four unique siRNA duplexes, targeting each of 1,006 unique human genes (Fig. 1C). The library consisted predominantly of nuclear enzymes, which we reasoned were more likely to function directly in the DDR and be targetable. Results of the primary screen were ranked by PCA score (Supplementary Table S1). The top 15% of these genes (156 genes) included 55 genes linked to the DDR (Fig. 1D; Supplementary Fig. S2; and Supplementary Table S2) including well characterized ATR signaling pathway genes *ATR*, *CHK1*, *RAD9*, *RAD1*, and *HUS1* and nucleotide metabolism genes *RRM1* and *RRM2*, known to regulate gemcitabine sensitivity (28), demonstrating that our screen can yield DDR genes that determine gemcitabine sensitivity.

CHD7 knockdown causes gemcitabine sensitization

Sixty eight of our hits were identified in previously published DNA damage sensitivity screens (17, 20, 29 36) and 27 are putative ATM/ATR substrates (17; Supplementary Table S3). We used these criteria to validate 47 of the 99 hits not characterized in the DDR in a secondary screen using deconvoluted individual siRNAs to confirm their gemcitabine sensitivity and eliminate false positives due to off target effects, and 38 of these genes induced gemcitabine sensitivity in at least two out of four siRNAs tested, including *CHD7* (Supplementary Table S4). Four of 4 siRNAs targeting CHD7 caused gemcitabine sensitization (Fig. 2A). Western blot analysis confirmed decreased levels of CHD7 following siRNA knockdown as well as specificity of the anti CHD7 antibody used for IHC analysis (Fig. 2B). A similar gemcitabine sensitization after CHD7 silencing was observed using a range of gemcitabine concentrations and in BxPC 3 and HPAC pancreatic cancer cells, suggesting that the phenotype is not cell type specific (Supplementary Fig. S3A C). CHD7 silencing in the absence of gemcitabine treatment reduced cell viability (Supplementary Fig. S3D). We also determined the gemcitabine sensitivity of CHD7 depleted cells using a colony formation assay. MIA PaCa 2 cells silenced for CHD7 demonstrated a significantly reduced percentage of surviving colonies following a 24 hour pulse of gemcitabine in a dose dependent manner compared with a NT control (Fig. 2C), confirming the gemcitabine sensitization of CHD7 depleted cells observed with WST 1 reagent. Consistent with these findings, MIA PaCa 2 and BxPC3 pancreatic cancer cells, which express lower levels of CHD7 than HPAC, CAPAN 1, and AsPC 1 pancreatic cancer cells, demonstrated increased gemcitabine sensitivity (Fig. 2D and E), suggesting that CHD7 expression may predict response to gemcitabine in pancreatic adenocarcinoma cells. To determine if CHD7 silencing causes gemcitabine sensitization of pancreatic cancer tumors *in vivo*, we generated a xenograft model using MIA PaCa 2 cells stably expressing shCHD7 or shControl (Fig. 2F). CHD7 silencing significantly delayed tumor growth in mice treated with gemcitabine compared with a control treated with gemcitabine (Fig. 2G), suggesting that CHD7 silencing also causes gemcitabine sensitization *in vivo*. No significant difference in body weight was observed in mice bearing tumors with shCHD7 compared with shControl and treated with or without gemcitabine (Supplementary Fig. S4).

CHD7 is a DDR protein

The gemcitabine hypersensitivity of CHD7 depleted cells suggests that CHD7 may function in the DDR. CHD7 silencing significantly increased the percentage of cells staining with γH2AX, a marker for DNA damage, following treatment with gemcitabine (Fig. 3A), suggesting that CHD7 silencing potentiates gemcitabine induced DNA damage. However, no significant difference in repair kinetics was observed between cells silenced with CHD7 compared with a nontargeting siRNA (Fig. 3A). CHD7 silenced cells showed a decreased percentage of cells in S phase and an increased percentage of cells in G_2 M in the absence of gemcitabine treatment (Supplementary Fig. S5A); however, no significant difference in cell cycle profile was observed between CHD7 depleted compared with NT control cells following gemcitabine treatment (Supplementary Fig. S5B). There was also no significant difference in protein levels of CHD7 in response to gemcitabine treatment (Supplementary Fig. S6). To determine whether CHD7 functions in ATR dependent signaling in response to gemcitabine treatment, we examined cells for the phosphorylation of CHK1 Ser317. CHD7 silencing significantly reduced CHK1 Ser317 phosphorylation but not total CHK1 protein levels in response to gemcitabine treatment (Fig. 3B), suggesting that CHD7 functions in controlling ATR dependent phosphorylation of CHK1 in response to gemcitabine treatment.

CHD7 is dysregulated and differentially expressed in pancreatic adenocarcinoma

Genes validated by our secondary screen or linked to the DDR were then analyzed for dysregulation and differential expression in pancreatic adenocarcinoma by mining of published data sets to determine their potential as biomarkers. Of these, eight genes demonstrate aberrant expression or somatic mutations in pancreatic adenocarcinoma (Fig. 3C and Supplementary Table S3) as reported in The Compendium of Potential Biomarkers (20) and the COSMIC database (11). Twelve of the genes are above the 90th percentile in differential

expression among a panel of 22 pancreatic adenocarcinoma cell lines or 20 pancreatic adenocarcinoma tissue samples (Fig. 3C and Supplementary Table S3; refs. 21, 22). Four of the genes exhibit both dysregulation and differential expression, including *CHD7*, which was selected for further analysis as a biomarker.

Survival analyses

Patient demographics, pathologic and treatment characteristics can be seen in Table 1. CHD7 expression was low in 84.7% of patients. Median tumor size was 3.4 cm (range, 1–6 cm), and 60% of patients were node positive. In addition to CHD7 expression, significant covariates on univariate analysis included tumor size, margin status, lymph node status, perineural invasion (PNI), lymphovascular invasion (LVI), and grade ($P < 0.2$). On Kaplan Meier analysis for patients receiving gemcitabine as a component of adjuvant therapy ($n = 42$), low CHD7 expression was associated with increased RFS (15 vs. 7 months; $P = 0.025$; Fig. 4A) and increased OS (18 vs. 10 months; $P = 0.015$; Fig. 4B). On multivariate analysis (Table 2), low CHD7 expression remained associated with increased RFS [HR, 0.12; 95% confidence interval (CI), 0.04–0.42; $P = 0.001$] and increased OS (HR, 0.09; 95% CI, 0.03–0.29; $P < 0.0001$). In the subset of patients receiving adjuvant therapy with agents other than gemcitabine [most commonly 5 fluorouracil (5 FU)], CHD7 was not associated with RFS ($P = 0.1$, data not shown) or OS ($P = 0.4$, data not shown). On Kaplan Meier analysis for all patients ($n = 59$), low CHD7 expression via IHC scoring was associated with increased RFS (15 months vs. 7 months; $P = 0.015$; Fig. 4C) and increased OS (19.5 months vs. 9 months; $P = 0.001$; Fig. 4D). These results remained significant on multivariate analysis (Table 3). To ensure stability of the multivariate model given the small number of events, the three least significant factors on univariate analysis were removed from the model (grade, PNI, and LVI) and the significance of CHD7 expression remained unchanged.

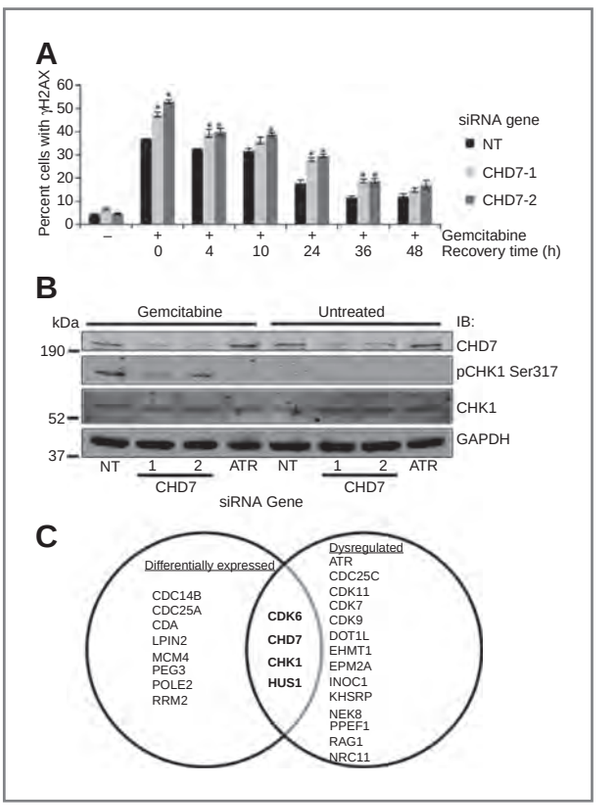

Figure 3. CHD7 is a DDR protein. A, MIA PaCa-2 cells were treated with or without gemcitabine for 20 hours, washed, released for the indicated time points, and processed for γH2AX staining by indirect immunofluorescence. The percentage (mean and SD) of γH2AX positive cells from three replicas is shown; *, $P < 0.05$. B, Western blot analysis of cell lysate from MIA PaCa-2 cells treated with or without gemcitabine for 6 hours and probed with anti-CHD7, pCHK-1 Ser317, CHK1, and GAPDH antibodies. C, Venn diagram showing gemcitabine sensitivity genes dysregulated and/or above the 90th percentile in differential expression among a panel of pancreatic cancer cell lines and tissue samples.

Table 1. Patient demographics, tumor characteristics, and treatment characteristics for all patients ($N = 59$)

	Median (range)	*N*	Percentage
Patient demographics			
Male sex		31	52.5
Ethnicity			
Asian		2	3.4
Black		12	20.3
White		42	71.2
Age (y)	60.0 (37–84)		
OS (mo)	17.3 (4.8–114.6)		
RFS (mo)	14.5 (0.6–109.8)		
Tumor characteristics			
Positive margins		20	24.5
Grade			
Well differentiated		5	6.3
Moderately differentiated		46	57.5
Poorly differentiated		28	35.0
Positive nodes		48	60.0
PNI		70	87.5
LVI		38	47.5
Low CHD7 expression		50	84.7
Tumor size (cm)	3.4 (1–6)		
Treatment characteristics			
Neoadjuvant therapy		2	3.4
Radiation therapy		39	66.1
Received gemcitabine		42	69.5

Discussion

In this study, we demonstrate a rationale driven approach for identifying novel biomarkers for outcome in patients with early stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine. Using a synthetic lethal screen to identify genetic determinants of gemcitabine sensitivity in human pancreatic cancer cells, we identified 93 genes that, when

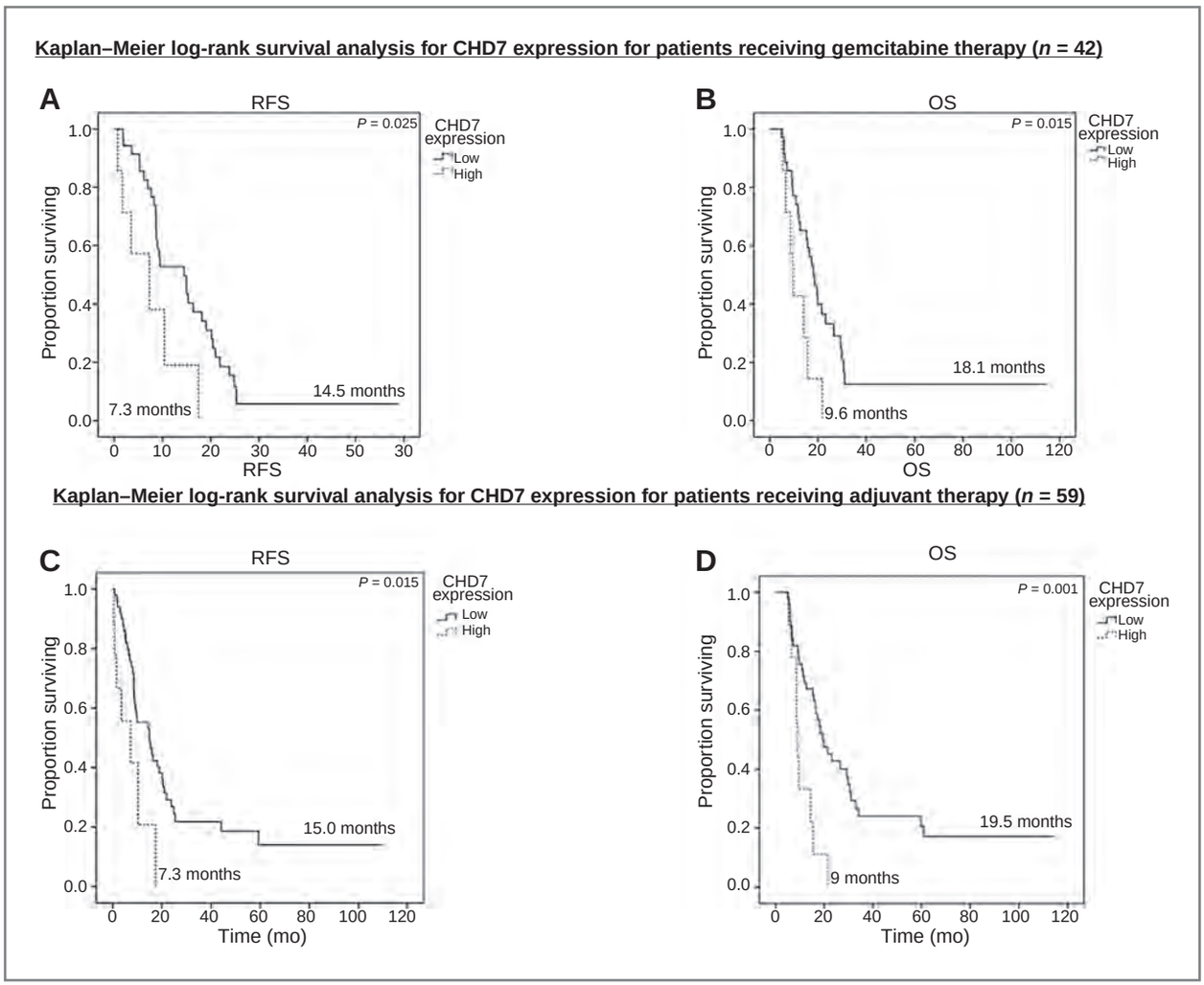

Figure 4. Kaplan Meier log rank survival analysis for CHD7 expression in patients receiving adjuvant therapy ($n = 59$) and in patients receiving gemcitabine therapy ($n = 42$). A, effect of CHD7 expression on RFS in patients receiving gemcitabine therapy ($n = 42$). B, effect of CHD7 expression on OS in patients receiving gemcitabine therapy ($n = 42$). C, effect of CHD7 expression on RFS in patients receiving adjuvant therapy ($n = 59$). D, effect of CHD7 expression on OS in patients receiving adjuvant therapy ($n = 59$).

silenced, demonstrate gemcitabine sensitization, including *CHD7*. CHD7 deficiency caused gemcitabine sensitization in pancreatic adenocarcinoma cells and delayed pancreatic tumor xenograft growth in mice treated with gemcitabine. We further found that CHD7 knockdown impaired ATR dependent phos phorylation of CHK1 and increased DNA damage induced by gemcitabine, revealing a novel function for CHD7 as a DDR protein, which maintains genome integrity in response to gemcitabine. We examined CHD7 as a potential biomarker based on its dysregulation and differential expression in a panel of pancreatic adenocarcinoma cell lines and tissues. Finally, we found that low CHD7 expression is associated with improved RFS and OS in patients with early stage resected pancreatic adenocarcinoma treated with adjuvant gemcitabine. These findings support our rationale driven approach in exploiting dysregulated DDR pathways in pancreatic adenocarcinoma to identify genetic determinants of gemcitabine sensitivity that can be translated to novel biomarkers or drug targets.

A third of the genes identified in our primary gemcitabine sensitivity screen are linked to the DDR, including ATR sig naling pathway genes *ATR*, *CHK1*, *RAD9*, *RAD1*, *HUS1*, and *CDK9* (37) and nucleotide metabolism genes *RRM1* and *RRM2*. *CHD7* was previously identified as a putative ATM/ATR sub strate (17). Our finding that *CHD7* silencing in human pan creatic cancer cells potentiates gemcitabine induced DNA damage and impairs CHK1 Ser317 phosphorylation in response to gemcitabine treatment suggests that CHD7 also functions in the ATR signaling pathway and helps to explain at least in part why CHD7 knockdown causes gemcitabine sen sitization in cells and *in vivo*. Still, the cell cycle effects of CHD7 expression require further understanding through future stud ies, which remain ongoing. For example, CHK1 inhibition has been shown to potentiate gemcitabine induced cytotoxicity by inducing premature mitosis (38). A number of genes identified in our screen, including *RRM1*, *RRM2*, and *CHK1* have previ ously been shown to determine gemcitabine sensitivity in

Table 2. Multivariate[a] Cox regression analyses for patients receiving gemcitabine therapy ($N = 42$)

	RFS			OS		
Outcome	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Tumor size	1.708	1.223–2.387	**0.002**	1.685	1.171–2.425	**0.005**
Positive margins	0.252	0.083—0.767	**0.015**	0.187	0.059–0.590	**0.004**
Higher grade	1.126	0.566–2.242	0.735	1.064	0.555–2.040	0.851
Positive nodes	1.177	0.5402–0.564	0.682	0.965	0.420–2.216	0.933
PNI	0.574	0.210–1.567	0.279	4.133	1.044–16.358	**0.043**
LVI	1.004	0.462–2.180	0.992	1.429	0.619–3.298	0.402
Low CHD7 expression	0.122	0.035–0.420	**0.001**	0.086	0.025–0.292	<**0.0001**

NOTE: Bold denotes statistical significance.

[a]Multivariate analysis includes all clinically relevant covariates with $P < 0.2$ on univariate analysis.

human pancreatic cancer cells (39), and low RRM2 expression has been shown to be associated with improved outcome in patients with pancreatic adenocarcinoma (24) and specifically those treated with adjuvant gemcitabine (28), providing val idation for our screen in identifying gemcitabine sensitivity genes that may function as potential biomarkers. Several of the gemcitabine sensitivity genes, including *PLK*1 and *AURKB*, are involved in mitotic progression that is in part targeted by nanoparticle albumin bound *(nab)* paclitaxel (Abraxane; Cel gene), which potentiates gemcitabine sensitivity and improves survival in patients with metastatic pancreatic adenocarcino ma treated with gemcitabine (40, 41). It is thus possible that the gemcitabine sensitivity genes reported in this study may also be novel druggable targets to be used in combination with gemcitabine. Indeed, *PARP2*, a target of PARP inhibitors that sensitizes pancreatic cancer cells to gemcitabine (42, 43), was also identified in our screen.

In our clinical data, low CHD7 expression was associated with increased OS and RFS in all patients receiving adjuvant therapy, although this was likely driven by the inclusion of patients receiving gemcitabine. The association of low CHD7 expression with increased survival was magnified in patients receiving gemcitabine as a component of their adjuvant ther apy despite smaller patient numbers, indicating that low CHD7 expression may indeed be associated with gemcitabine sensi tivity in these patients. In contrast, CHD7 expression in patients not receiving gemcitabine was not statistically signif icant. This analysis is underpowered with limitation of small sample size and selection bias, but our findings provide valuable hypothesis generating data suggesting that CHD7 may have predictive value in these patients.

Given the evidence that patients with low CHD7 expression demonstrate improved outcomes, it is possible that adjuvant therapy regimens could be tailored to individualize patient treatment based on CHD7 expression. This should be exam ined in future prospective trials and in larger secondary analyses of completed prospective studies. Although adjuvant chemotherapy for patients with pancreatic adenocarcinoma is advantageous, the ideal drug regimen remains unclear. The benefit of adjuvant gemcitabine compared with adjuvant 5 FU in patients with early stage resected pancreatic adenocarci noma has not been demonstrated in any large trials. Both the ESPAC 3 trial, which randomized patients with resected pan creatic adenocarcinoma to adjuvant gemcitabine versus 5 FU, and the RTOG 97 04 trial, which randomized patients with resected pancreatic adenocarcinoma to adjuvant pre and postchemoradiotherapy gemcitabine versus 5 FU, reported no significant difference in disease free survival (DFS) or OS

Table 3. Multivariate[a] Cox regression analyses for patients receiving adjuvant therapy ($N = 59$)

	RFS			OS		
Outcome	**HR**	**95% CI**	***P***	**HR**	**95% CI**	***P***
Tumor size	1.622	1.228–2.144	**0.001**	1.527	1.130–2.063	**0.006**
Positive margins	0.849	0.408–1.768	0.662	0.724	0.347–1.511	0.389
Higher grade	1.051	0.579–1.907	0.870	1.057	0.592–1.887	0.851
Positive nodes	1.638	0.777–3.452	0.194	1.729	0.818–3.652	0.151
PNI	0.410	0.161–1.044	0.062	2.028	0.591–6.960	0.261
LVI	1.064	0.529–2.139	0.862	1.082	0.537–2.178	0.826
Low CHD7 expression	0.271	0.107–0.687	**0.006**	0.203	0.085–0.486	<**0.0001**

NOTE: Bold denotes statistical significance.

[a]Multivariate analysis includes all clinically relevant covariates with $P < 0.2$ on univariate analysis.

between the two arms (3, 44). Our finding that low CHD7 expression is associated with improved outcome in patients with early stage pancreatic adenocarcinoma treated with adjuvant gemcitabine suggest that, once validated, CHD7 expression could potentially be used as a predictive biomarker to individualize adjuvant therapy for these patients. In addition, the optimal radiation dose and fractionation for patients with resected pancreatic adenocarcinoma remains unknown, and molecular biomarkers to guide adjuvant therapy decisions are essential (45). The potential utility of CHD7 expression as a prognostic and potentially predictive biomarker still remains a hypothesis generating observation and requires validation in a prospective clinical trial, in which regimen dosing and duration are more homogenous.

Interest in genetic sequencing data, such as with The Cancer Genome Atlas and other similar projects, continues to increase (46, 47), leading to rapidly increasing knowledge of genes expressed and mutated in specific cancer types including pancreatic adenocarcinoma. As this knowledge becomes available, it is crucial that an approach be developed to help identify those genes that may serve as clinically relevant prognostic or predictive biomarkers or potential drug targets for novel therapeutics. The successful identification and validation of *CHD7* as a novel gemcitabine sensitivity gene that is associated with outcome in patients with early stage pancreatic adenocarcinoma treated with adjuvant gemcitabine is evidence that our approach may be successful in identifying other clinically relevant biomarkers or drug targets.

It is worth noting that recent advances in chemotherapy have increased the use of FOLFIRINOX therapy in the metastatic setting, impacting the potential utility of this study. Still, National Comprehensive Cancer Network guidelines in the metastatic setting equivalently recommend FOLFIRINOX or two gemcitabine based regimens (gemcitabine with the addition of erlotinib or nab paclitaxel), both with category one evidence (24). In addition, gemcitabine or fluoropyrimidine therapies are still recommended in the adjuvant setting, which is where this study's clinical focus remains. Future studies should evaluate the predictive role of CHD7 in a larger, randomized prospective trial to validate potential gemcitabine sensitivity genes in a similar fashion to other identified predictive biomarkers (48). The current study suggests that CHD7 may be a useful biomarker for determining which patients will derive greater benefit from gemcitabine therapy, providing clinicians a way to better select patients for specific adjuvant therapy regimens in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

Low CHD5 expression activates the DNA damage response and predicts poor outcome in patients undergoing adjuvant therapy for resected pancreatic cancer

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The DNA damage response (DDR) promotes genome integrity and serves as a cancer barrier in precancerous lesions but paradoxically may promote cancer survival. Genes that activate the DDR when dysregulated could function as useful biomarkers for outcome in cancer patients. Using a siRNA screen in human pancreatic cancer cells, we identified the *CHD5* tumor suppressor as a gene, which, when silenced, activates the DDR. We evaluated the relationship of CHD5 expression with DDR activation in human pancreatic cancer cells and the association of CHD5 expression in 80 patients with resected pancreatic adenocarcinoma (PAC) by immunohistochemical analysis with clinical outcome. CHD5 depletion and low CHD5 expression in human pancreatic cancer cells lead to increased H2AX-Ser139 and CHK2-Thr68 phosphorylation and accumulation into nuclear foci. On Kaplan–Meier log-rank survival analysis, patients with low CHD5 expression had a median recurrence-free survival (RFS) of 5.3 vs 15.4 months for patients with high CHD5 expression ($P=0.03$). In 59 patients receiving adjuvant chemotherapy, low CHD5 expression was associated with decreased RFS (4.5 vs 16.3 months; $P=0.001$) and overall survival (OS) (7.2 vs 21.6 months; $P=0.003$). On multivariate Cox regression analysis, low CHD5 expression remained associated with worse OS (HR: 3.187 (95% CI: 1.49–6.81); $P=0.003$) in patients undergoing adjuvant chemotherapy. Thus, low CHD5 expression activates the DDR and predicts for worse OS in patients with resected PAC receiving adjuvant chemotherapy. Our findings support a model in which dysregulated expression of tumor suppressor genes that induce DDR activation can be utilized as biomarkers for poor outcome.

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Keywords: CHD5; DNA damage response; biomarker; pancreatic cancer; tumor suppressor

INTRODUCTION

Pancreatic adenocarcinoma (PAC) is a devastating malignancy, and 5-year overall survival (OS) rates for all patients remain less than 10%.[1] Despite large, prospective randomized trials, the optimal management of PAC following surgical resection is still unknown. Several trials have resulted in equivocal conclusions regarding the optimal use of various combinations of adjuvant chemotherapy and radiation therapy or chemotherapy alone.[2–4] Currently the data supporting prognostic biomarkers in PAC continue to evolve, which make optimal treatment decisions difficult. Given the increasingly apparent genetic heterogeneity of PAC, it seems conceivable that genetic profiles exist to provide prognostic information, which could help to predict response to adjuvant therapy.[5–8]

The DNA damage response (DDR) is critical for the maintenance of genome integrity and serves as a cancer barrier by mobilizing DNA repair, cell cycle arrest or apoptosis.[9,10] In human precancerous lesions, replication stress resulting from activated oncogenes and inactivated tumor suppressor proteins induces activation of the DDR, which constrains tumor development. This creates a selection pressure for mutations or epigenetic silencing of DDR genes leading to the proliferation of genetically unstable cells and ultimately resulting in cancer. Thus, the DDR acts as a barrier against genomic instability and cancer development. Indeed, a large number of DDR genes are somatically mutated in PAC, including *ATM*, *BRCA2*, *CDKN2A*, *FANCI*, *HELB* and *RAD9*.[11] Although the DDR promotes genome integrity and helps to prevent progression of precancerous lesions, it may paradoxically promote the survival of PAC that outgrow the selection pressure of DDR activation. These cancers could potentially be more resistant to the replication stress and DNA damage induced by chemotherapy and radiation therapy that is used in the adjuvant setting for early-stage PAC. The clinical significance of dysregulated expression of genes that induce DDR activation in PAC and other cancers is currently not known.

Chromodomain helicase DNA-binding protein 5 (CHD5) is a member of a family of chromodomain enzymes that belong to the ATP-dependent chromatin remodeling protein SNF2 superfamily and characterized as a tumor suppressor. CHD5 has been shown to be mutated, deleted or silenced in a number of malignancies including neuroblastoma, gastric cancer, leukemia/lymphoma, melanoma, breast, prostate, ovarian and lung cancers, squamous cell of the larynx and gliomas.[12–24] The most thorough characterization of CHD5 is in neural tissues where it controls proliferation, apoptosis and senescence via the p19(Arf)/p53

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pathway.[12] CHD5 binds to histone H3 for its tumor suppressor function.[25] CHD5 is also a putative substrate of the ATM/ATR checkpoint kinases, suggesting that it may have a role in the DDR.[26] Furthermore, low CHD5 expression has been shown to correlate with poor patient outcomes in a number of other malignancies, independently of other known prognostic factors.[21,24,27–29] The role of CHD5 expression in PAC has not previously been explored.

RESULTS

CHD5 silencing activates the DDR

To identify genes, which, when dysregulated, induce DDR activation, we completed a loss-of-function genetic screen in MIA PaCa-2 human pancreatic cancer cells for genes, which when silenced increase DDR signaling. Our library included 200 siRNAs targeting 50 nuclear enzyme genes. MIA PaCa-2 cells were transfected with siRNA and analyzed 72 h later by indirect immunofluorescence microscopy for phosphorylation of H2AX Ser139 (γH2AX), an ATM/ATR substrate and early marker of DDR activation. Two siRNAs targeting a different region of CHD5 significantly induced γH2AX foci formation above a non-targeting control (Figures 1a and b). Similarly two siRNAs targeting CHD5 significantly induced formation of phospho-CHK2 Thr68 foci (Figures 1c and d), an ATM/ATR substrate and second marker of DDR activation, compared with a non-targeting control. Western blot analysis confirmed knockdown of CHD5 expression, specificity

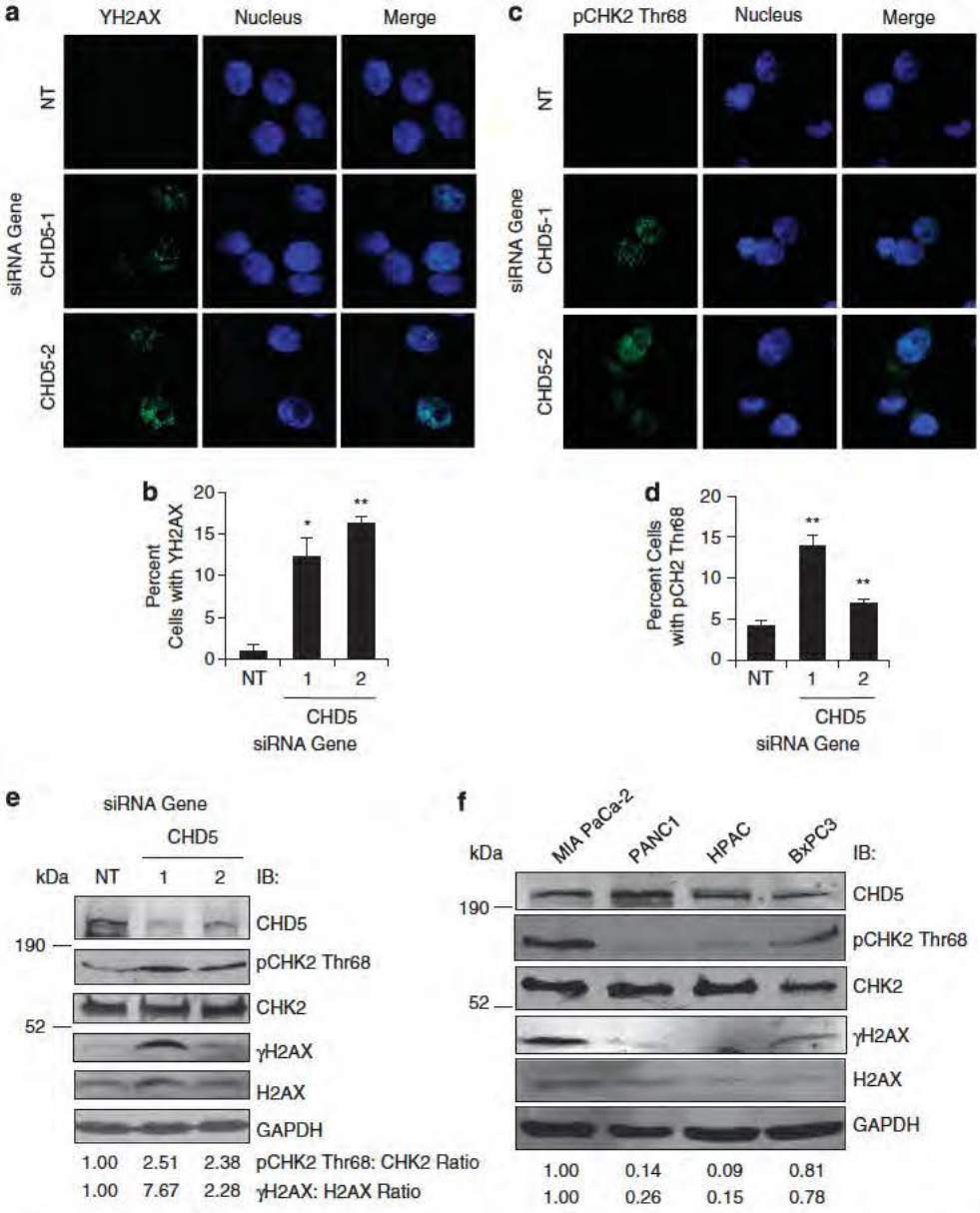

Figure 1. CHD5 silencing activates the DDR. (**a**) MIA PaCa-2 cells were transfected with NT or CHD5 siRNA and processed 72 h later for γH2AX staining by indirect immunofluorescence microscopy. (**b**) The percentage (mean and s.e.m.) of γH2AX foci-positive cells from three replicate experiments is shown. $*P<0.05$. $**P<0.01$. (**c**) MIA PaCa-2 cells were transfected with NT or CHD5 siRNA and processed 72 h later for phospho-CHK2 (pCHK2) Thr68 foci staining by indirect immunofluorescence microscopy. (**d**) The percentage (mean and s.e.m.) of pCHK2 Thr68 foci-positive cells from three replicate experiments is shown. $*P<0.05$. $**P<0.01$. (**e**) MIA PaCa-2 cells were transfected with NT or CHD5 siRNA, collected 72 h later, separated by SDS-PAGE and immunoblotted with antibodies against CHD5, pCHK2 Thr-68, total CHK2, γH2AX, total H2AX and GAPDH. (**f**) Western blot analysis of cell lysate from MIA PaCa-2, PANC1, HPAC and BxPC3 cells with the indicated antibodies. For (**e**) and (**f**), pCHK2 Thr-68: total CHK2 and γH2AX:total H2AX of representative blot from three independent experiments is shown.

of the anti-CHD5 antibody, as well as increased phosphorylation of CHK2 Thr68 and γH2AX (Figure 1e). Consistent with these findings, MIA PaCa-2 and BxPC3 pancreatic cancer cells, which express lower levels of CHD5 than PANC1 and HPAC pancreatic cancer cells, demonstrated increased phosphorylation of CHK2 Thr68 and γH2AX (Figure 1f). Collectively, these findings suggest that CHD5 silencing and low CHD5 expression in cells activates the DDR.

To determine whether CHD5 expression might function as an ideal biomarker, we mined published genome-wide data sets to determine if its expression is differentially expressed among pancreatic cancer cell lines and PAC patient samples. CHD5 was found to be above the 90th percentile of genes for differential expression among a panel of 22 PAC cell lines and 20 PAC patient samples.[30,31]

Clinical results

Patient characteristics can be seen in Table 1. Median patient age was 63.1 years (37–84), and median follow-up for survivors was 53 months (range 6–114). The median OS for all patients was 15.4 months (range 2.8–114.6), and median RFS was 9.3 months (range 0.6–119.8). At last known follow-up, 14 (17.5%) patients had no evidence of disease, and 62 (77.5%) of patients were deceased. A total of 59 (73.8%) patients received adjuvant therapy, with most of these patients receiving gemcitabine-based regimens ($n=$ 41/59; 69.5%) and radiation therapy (39/59; 66.1%). Forty-two patients (52.5%) were male, and 59 (73.8%) were white. Tumor size ranged from 1 cm to 6 cm with a median of 3.3 cm; 20 patients (24.5%) had positive margins and 48 (60%) had positive lymph nodes.

Survival analyses: all patients

Eighty patients were analyzed in this group. Kaplan–Meier analyses showed an association between low CHD5 expression and decreased OS in all patients (8.5 months vs 18.1 months; $P=0.12$; Figure 2a) and recurrence-free survival (RFS) (5.3 months vs 15.4 months; $P=0.03$; Figure 2a). The complete results of the univariate and multivariate Cox regression analyses for all patients can be seen in Table 2. Low CHD5 expression was associated with decreased OS on multivariate analysis (HR: 2.3 (95% CI: 1.2–4.5); $P=0.01$). The only other significant prognostic factor on multivariate analysis was lymphovascular invasion (HR: 1.4 (95% CI: 0.6–3.4); $P=0.04$).

Subset analyses: patients receiving adjuvant therapy

In the subset of patients receiving adjuvant therapy, 59 patients were analyzed. Kaplan–Meier analyses showed an association between low CHD5 expression and decreased OS in patients undergoing adjuvant therapy (7.2 vs 21.6 months; $P=0.003$; Figure 2b) and RFS (4.5 vs 16.3 months; $P=0.001$; Figure 2b). This relationship persisted on multivariate analysis, examining the effect of CHD5 expression on OS (HR: 3.19 (95% CI: 1.5–6.81); $P=0.003$). The full multivariate results for patients receiving adjuvant therapy can be seen in Table 3.

As the initial exploratory intent of these genetic markers was to provide prognostic data for patients undergoing adjuvant gemcitabine-based therapy, a second subset analysis was performed in the patients receiving only gemcitabine-based therapy, and the previously defined relationship remained significant on multivariate analysis, with low CHD5 expression associated with decreased RFS (HR: 3.08 (95% CI: 1.09–8.73); $P=0.035$; Supplementary Table S1) and decreased OS (HR: 3.041 (95% CI: 1.13–8.16); $P=0.027$; Supplementary Table S1). Finally an exploratory multivariate analysis was performed comparing the prognostic significance of CHD5 with other known prognostic genetic markers, including excision repair cross-complementing gene 1 (ERCC1) and ribonucleotide reductase subunit 2 (RRM2) that have been previously published and shown to be significant using this patient cohort.[8] The results of this multivariate analysis can be seen in Table 4; of note, CHD5 remained significant independent of other known prognostic genetic markers in this population, ERCC1 and RRM2.[7,8]

Table 1. Patient demographics, tumor characteristics and treatment characteristics for all patients (N 80)

		N	*Percent*
Patient Demographics			
Age (years)	63.1 (37 84)[a]		
Male sex		42	52.5
Ethnicity			
Asian		2	2.5
Black		15	18.8
White		59	73.8
Overall survival (months)	15.4 (2.8 114.6)[a]		
Recurrence free survival (months)	9.3 (0.6 119.8)[a]		
Pathological characteristics			
Positive margins		20	24.5
Grade			
1		5	6.3
2		46	57.5
3		28	35.0
Positive nodes		48	60.0
Perineural invasion		70	87.5
Lymphovascular invasion		38	47.5
High CHD5 expression		63	78.75
Tumor size (cm)	3.3 (1 6)[a]		
Treatment characteristics			
Neoadjuvant therapy		2	2.5
Radiation therapy		39	48.8
Received gemcitabine		41	51.3

[a]Median (Range).

DISCUSSION

Our results demonstrate that silencing of the CHD5 tumor suppressor and low CHD5 expression in human pancreatic cancer cells lead to activation of the DDR and that low CHD5 expression is associated with worse RFS and OS in patients with PAC treated by resection and adjuvant chemotherapy. These findings support a model in which dysregulated expression of tumor suppressor genes that induce DDR activation can function as biomarkers for poor outcome.

The role and function of CHD5 have been explored in several malignancies, and CHD5 has been well characterized as a tumor suppressor protein.[12,21,23,29] Multiple studies have demonstrated that silencing or downregulation of CHD5 is associated with the development of various malignancies including lung,[22] gastric,[15] neuroblastoma,[23] breast,[21] and laryngeal squamous cell carcinoma.[32] We have presented unique data that demonstrate the prognostic ability of CHD5 for patients receiving adjuvant chemotherapy following resection for PAC. In addition to the clinically significant association of CHD5 expression, we have presented a mechanistic explanation as to the etiology of these clinical findings. Our preclinical data demonstrate that low CHD5 expression in human pancreatic cancer cells leads to activation of the DDR with increased γH2AX and phosho-CHK2 Thr68 foci accumulation and expression. We hypothesize that the increased activation of the DDR in cells with low CHD5 expression could contribute to the worse clinical outcomes seen in this

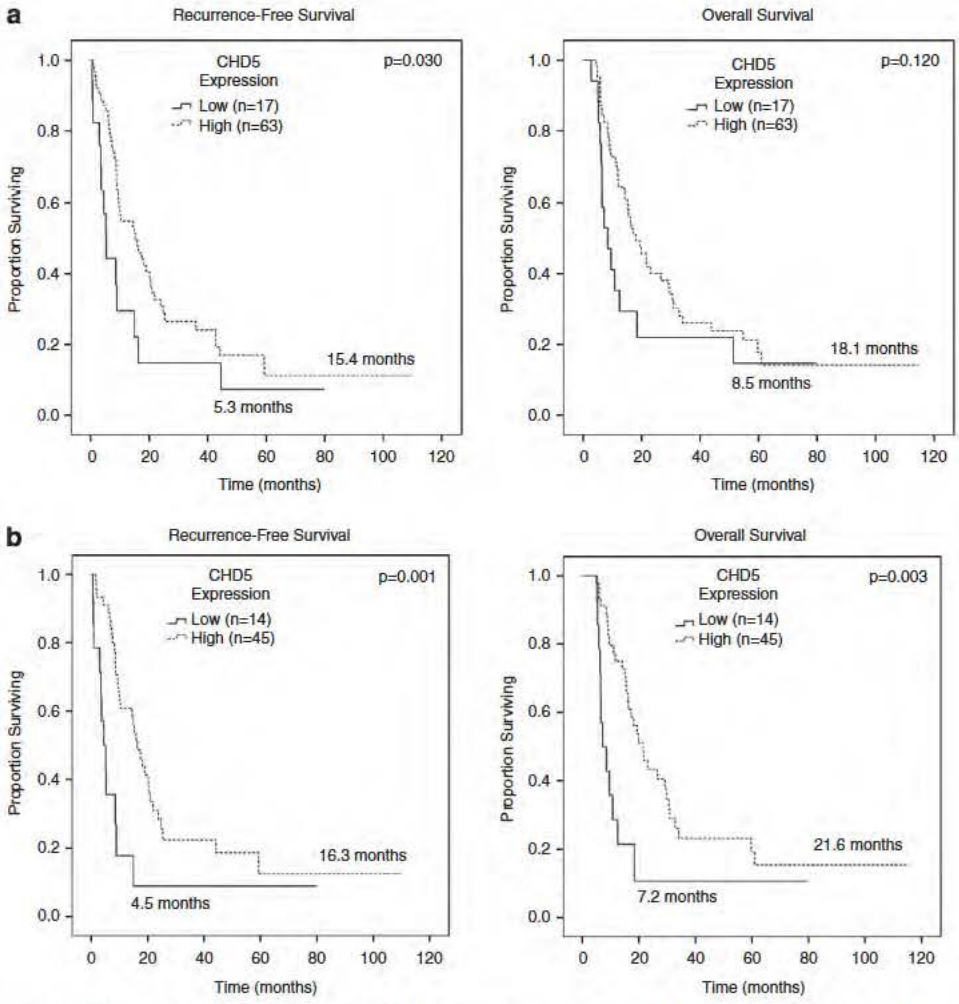

Figure 2. Kaplan Meier log-rank survival analysis examining CHD5 expression associated with OS and RFS in (**a**) all patients and (**b**) patients undergoing adjuvant therapy only.

Table 2. Univariate and multivariate[a] Cox regression analyses for all patients (*N* 80)

Outcome	*RFS*				*OS*			
	Univariate		*Multivariate*[a]		*Univariate*		*Multivariate*[a]	
	HR (95% CI)	P *value*	*HR (95% CI)*	P *value*	*HR (95% CI)*	P *value*	*HR (95% CI)*	P *value*
Tumor size	1.3 (1.0 1.6)	0.02	1.3 (1.0 1.3)	0.02	1.3 (1.0 1.6)	0.02	1.2 (1.0 1.6)	0.1
Positive margins	1.8 (1.1 3.0)	0.02	1.4 (0.8 2.5)	0.2	1.7 (1.1 2.8)	0.03	1.3 (0.7 2.2)	0.4
Positive nodes	1.8 (1.0 3.0)	0.04	1.5 (0.8 2.9)	0.2	2.0 (1.1 3.4)	0.01	1.5 (0.8 2.9)	0.2
PNI	1.0 (0.5 2.1)	0.9	0.9 (0.4 2.0	0.8	3.9 (1.2 12.4)	0.02	3.8 (1.2 12.7)	0.03
LVI	1.5 (.9 2.5)	0.1	1.4 (0.8 2.5)	0.3	2.0 (1.2 3.3)	0.01	1.4 (0.6 3.4)	0.04
Higher grade	1.3 (0.9 2.0)	0.2	1.3 (0.8 2.0)	0.3	1.3 (0.8 1.9)	0.3	1.7 (0.9 3.0)	0.08
Low CHD5 expression[b]	2.0 (1.0 3.3)	0.03	2.5 (1.3 4.6)	0.006	1.6 (0.8 2.9)	0.1	2.3 (1.2 4.5)	0.01

Abbreviations: LVI, lymphovascular invasion; OS, overall Survival; PNI, perineural invasion; RFS, recurrence free survival. [a]Multivariate analysis includes all clinically relevant covariates and covariates with *P* value < 0.1 on univariate analysis. [b]CHD5 Score is decreasing from high expression to low expression.

patient cohort. The dysregulation of CHD5 expression and activation of the DDR could promote the survival of PAC that outgrow the selection pressure of DDR activation through compensatory DNA repair or pro-survival pathways. Specifically, these cancers may be more resistant to the replication stress and DNA damage induced by chemotherapy and radiation therapy that is used in the adjuvant setting for early-stage PAC, and thus contribute to the worse clinical outcomes seen in these patients.

CHD5 expression levels have been shown to be prognostic in various other disease sites. In a series by Wong *et al.*, down-regulation of CHD5 expression was shown to be an independent adverse prognostic factor in patients with ovarian cancer. Similar

Table 3. Multivariate[a] Cox regression analyses for adjuvant therapy patients (*N* 59)

Outcome	*RFS*			*OS*		
	HR	*95% CI*	P *value*	*HR*	*95% CI*	P *value*
Tumor size	1.411	1.095 1.817	0.008	1.276	0.967 1.684	0.085
Positive margins	0.999	0.488 2.043	0.998	0.996	0.471 2.109	0.993
Higher grade	1.072	0.593 1.940	0.817	1.157	0.645 2.075	0.625
Positive nodes	1.964	0.910 4.236	0.085	1.770	0.841 3.726	0.133
PNI	0.488	0.191 1.252	0.136	1.790	0.541 5.919	0.340
LVI	1.078	0.543 2.138	0.830	1.346	0.673 2.691	0.401
Low CHD5 expression[b]	3.013	1.383 6.563	0.006	3.187	1.492 6.808	0.003

Abbreviations: LVI, lymphovascular invasion; OS, overall Survival; PNI, perineural invasion; RFS, recurrence free survival. [a]Multivariate analysis includes all clinically relevant covariates and covariates with *P* value < 0.1 on univariate analysis. [b]CHD5 Score is decreasing from high expression to low expression.

Table 4. Multivariate[a] Cox regression analyses for CHD5, ERCC1 and RRM2 in adjuvant patients (*N* 31)

Outcome	*RFS*			*OS*		
	HR	*95% CI*	P *value*	*HR*	*95% CI*	P *value*
Tumor size	1.253	0.930 1.687	0.138	1.214	0.889 1.658	0.223
Positive margins	1.624	0.730 3.614	0.235	1.456	0.635 3.337	0.374
Higher grade	1.654	0.858 3.187	0.133	1.363	0.750 2.479	0.310
Positive Nodes	1.573	0.750 3.297	0.231	1.360	0.642 2.880	0.422
PNI	0.575	0.219 1.508	0.260	1.944	0.587 6.431	0.276
LVI	1.256	0.639 2.471	0.508	1.377	0.694 2.733	0.361
Low CHD5 expression[b]	3.777	1.736 8.217	**0.001**[c]	2.672	1.234 5.784	**0.013**
High RRM2 expression	3.173	1.118 9.000	**0.030**	1.319	0.431 4.036	0.627
High ERCC1 expression	9.058	3.36724.369	**<0.0001**	7.173	2.673 19.247	**<0.0001**

Abbreviations: ERCC, excision repair cross complementing gene 1; LVI, lymphovascular invasion; OS, overall Survival; PNI, perineural invasion; RFS, recurrence free survival; RRM2, ribonucleotide reductase subunit 2. [a]Multivariate analysis includes all clinically relevant covariates with *P* value <0.2 on univariate analysis. [b]CHD5 score is decreasing from high expression to low expression. [c]Bold font denotes statistical significance.

to the current study, the decreased expression of CHD5 was prognostic independent of other clinically significant variables such as age, tumor type, grade and clinical stage.[28] In a second series by Du *et al.*, low CHD5 expression was prognostic for decreased RFS and OS in patients with primary gallbladder carcinoma when compared with patients with high CHD5 expression. Again, CHD5 expression was a predictor of poor outcomes independent of other known prognostic factors.[33] In a third series by Garcia *et al.*, the expression of CHD5 was again shown to be a marker of outcome in patients with neuroblastoma independent of other relevant biological or clinical parameters. In the Garcia series, higher CHD5 expression was associated with an improved OS and event-free survival. It was also shown that after induction chemotherapy, reactivation of CHD5 expression was correlated with prolonged OS and improved clinical response. It appears consistent across a variety of malignancies that decreased CHD5 expression is associated with worse clinical outcomes.

The current series presents a cohort of 80 patients in whom CHD5 was associated with a statistically significant RFS difference but not statistically significant OS difference on KM analysis when analyzed in the cohort as a whole. This lack of significance for OS may be a consequence of the relatively small number of patients with low CHD5 expression and limitation of insufficient statistical power. However, when the analysis was repeated excluding the patients who did not receive adjuvant therapy, CHD5 became a highly significant prognostic factor for both OS and RFS, indicating that there may be a stronger correlation in this group. This finding is consistent with the hypothesis that CHD5 expression levels are correlated with cells' ability to respond to or repair DNA damage in patients who receive adjuvant therapy.

CHD5 function has been explored primarily in the central nervous system (CNS). The tumor suppressor activity of CHD5 has been shown to be mediated through the p19arf/p53 pathway.[12] CHD5 was also found to be a putative ATM/ATR substrate.[26] Our finding that CHD5 silencing in cells activates the DDR provides support for CHD5 as a genome maintenance protein that responds to replication stress. The rationale is that, in the absence of exogenous DNA damage, the DDR will be activated as a result of spontaneous DNA damage resulting from collapsed replication forks.

Given the poor prognosis of PAC even following R0 surgical resection, genetic markers that could provide additional prognostic information for these patients are critical. Such genetic markers may help identify subsets of PAC patients at particularly high risk for poor outcomes. Improving our ability to identify such patients will help to identify those patients who may benefit the most from aggressive therapy. With further validation, given that patients with low CHD5 expression have poor outcomes, it is possible that adjuvant therapy regimens could be tailored to individualize patient treatment based on CHD5 expression. Patients with low CHD5 expression for instance may benefit from participation in clinical trials of novel therapeutic regimens, as they are less likely to benefit from standard adjuvant treatment.

The current study is the first to demonstrate the prognostic significance of CHD5 expression in patients with resected PAC. Further study of CHD5 expression in patients with resected PAC, including validation in a prospective randomized trial, is needed to better characterize the prognostic significance of this gene. In addition, CHD5 warrants further analysis of its function in the DDR given its clinical relevance as both a tumor suppressor and prognostic factor in a number of malignancies.

MATERIALS AND METHODS

Cell culture and transfections

Human MIA PaCa 2, PANC1, HPAC and BxPC3 pancreatic cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown under standard cell culture conditions. siRNA duplexes were obtained from Dharmacon (Pittsburgh, PA, USA) and transfected with HiPerfect (Qiagen, Venlo, Limburg, Netherlands), according to the manufacturer's instructions.

Individual siRNA sequences are as follows:

non targeting (ATGAACGTGAATTGCTCAATT);
CHD5 1 (GGAAAGACCUGCCCUACGA); D 009878 01;
CHD5 2 (GAAAGAACGACAUGGAUGA); D 009878 04 renamed to CHD5 2.

Immunofluorescence

Seventy two hours after transfection, cells were washed with 1xPBS, fixed with 2% paraformaldehyde for 10 min at room temperature, permeabilized with 0.5% Triton X 100 (Fisher Scientific, Waltham, MA, USA) and blocked in a 5% bovine serum albumin solution (Sigma, St Louis, MO, USA). Cells were then immunostained with an anti phospho histone H2AX (Ser139) antibody (Millipore, Billerica, MA, USA, 05 636) and anti mouse secondary antibody with AlexaFluor 488 (Invitrogen, Carlsbad, CA, USA, A21202) or an anti phospho CHK2 (Thr68) antibody (Cell Signaling, Danvers, MA, USA, 2661) and anti rabbit secondary antibody with AlexaFluor 488 (Invitrogen, A21206). After incubation, slides were mounted onto slides with a mounting media containing 4′, 6 diamidino 2 phenylindole (DAPI) and dried. Analysis was performed using a Zeiss Observer Z1 (Carl Zeiss Microscopy, Jena, Germany) microscope with Axiovision Rel 4.8 software (Zeiss, Jena, Germany) using the ×63 oil objective. Foci quantitation was conducted by counting 100 healthy cells and scoring cells with 10 or more foci as positive. Experiments were done in triplicate. Images were processed using the Adobe Photoshop software (San Jose, CA, USA).

Western blot analysis

Seventy two hours after transfection, cells were collected in PBS and then lysed for 30 min on ice with NP40 buffer containing 200 mM NaCl, 1% NP40, 50 mM Tris HCl (pH 8.0), supplemented with fresh protease inhibitors. Samples were centrifuged at 13 000 r.p.m. for 10 min at 4 °C, afterward clear lysate was extracted. A Bradford assay was used to determine protein concentration, where in samples were loaded into a SDS PAGE gel, transferred to a PVDF membrane and subsequently probed with antibodies against CHD5 (Novus, 23320002), phospho CHK2 (Thr68) (Cell Signaling, 2661), phospho histone H2AX (Ser139) antibody (Millipore, 05 636), H2AX (Bethyl, Montgomery, TX, USA, A300 082A), CHK2 (Santa Cruz, Dallas, TX, USA, sc 17748) and GAPDH (GeneTex, Irvine, CA, USA, GTX627408) followed by LI COR IRDye secondary antibodies. Detection and densitometric analysis was performed using the Odyssey system. Images were processed using the Adobe Photoshop software.

Differential expression analysis

For determination of differential expression, we extracted gene expression data from the two Gene Expression Omnibus (GEO) submissions based on the Affymetrix U133 Plus 2.0 platform: (1) GSE12654: a 22 pancreatic cancer cell line study and (2) GSE16515: 20 pancreatic patient tumors.[30,31] Within each study, after processing and normalization, we performed a genome wide filter to identify genes with 'large' expression differences among tumors, and, separately, among cell lines, using a variance approach. We define 'differential expression' as genes whose expression variability is 'large' relative to all other genes on the array, where 'large' is defined according to whether expression variability associated with a gene was greater than the 90th percentile from all the genes. It was in this analysis that CHD5 emerged as highly statistically significant for further exploration in PAC.

Patient selection

A total of 80 patient specimens with adequate tissue available for analysis were obtained from a prospectively maintained surgical tissue bank of primary tumor samples. Each of patients included in the study had undergone pancreaticoduodenectomy or distal pancreatectomy for PDAC between the dates of January 2000 and October 2008. Analysis and genetic screens have been previously published using the same data set, and much of the methods for data acquisition have been previously reported.[7,8] RFS was measured based on surveillance imaging of the chest, abdomen and pelvis, obtained at regular intervals after resection. OS was determined based on the clinical follow up data available in each patient record and was measured from the day of surgery. Relevant prognostic factors for PAC were obtained from both clinical records and individual pathology reports. Institutional Review Board approval was obtained before any data review or analysis, and patient confidentiality was maintained according to the Health Insurance and Patient Accessibility Act.

Immunohistochemical analysis

The immunohistochemical (IHC) analysis was limited to primary tumor tissue and adjacent normal tissue. Slides that had been formalin fixed and paraffin embedded were reviewed by a staff pathologist to confirm the presence and quality of tumor. The tissue was stained using an anti CHD5 antibody (Novus, 23320002). Specificity of the anti CHD5 antibody was validated by western blot analysis following siRNA silencing (Figure 1e). The percentage of cell staining and intensity of staining were factored into a previously published scoring system.[8] A singe pathologist (BS) blinded to each of the patient outcomes reviewed each case. Fine granular nuclear staining was regarded as positive, and the percentage of cells that showed labeling was scored as 0 <1%, 1 1 10%, 2 11 50% and 3 51 100%. In addition to the total percentage of staining that was given, the intensity of the staining was scored as weak 1, moderate 2 or strong 3. For the cases that demonstrated heterogeneous staining, the dominant intensity pattern was used. Representative IHC staining for each of the staining groups can be found in Figure 3. The gene expression scoring system used has been previously published and gives more weight to the percentage of cells staining, as this is thought to be more biologically significant.[8] In general, for this scoring system, a score of $\leqslant 1$ was classified as low expression, whereas a score of >1 was classified as high expression.

Statistical analysis

Univariate and multivariate Cox regression analyses were performed for all patients to examine the impact of CHD5 expression levels on both OS and RFS. Kaplan Meier log rank survival analysis was performed for RFS and OS based on CHD5 expression levels. Factors that were significant to a level of $P<0.2$ on univariate analysis were included in the multivariate analysis; these included tumor size, margin status, nodal status, perineural invasion,

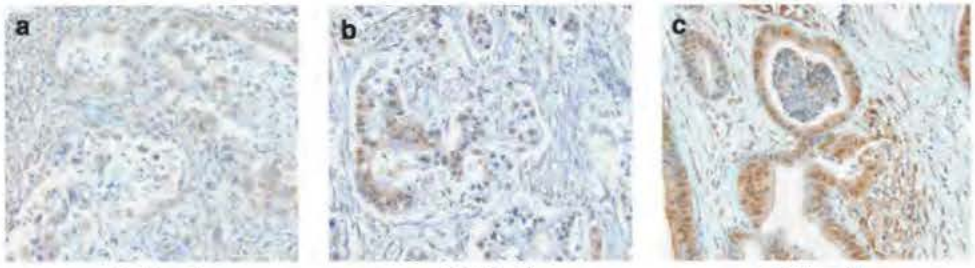

Figure 3. Representative immunohistochemical analysis for CHD5 expression in primary tumor tissue. The intensity of nuclear expression of CHD5 was graded as (**a**) weak - 1, (**b**) moderate - 2 or (**c**) strong - 3. Normal pancreatic tissue is also pictured with CHD5 staining characteristics showing islets and acini, demonstrating usually cytoplasmic positivity and staining of islets appearing more than acini. The duct epithelium show nuclear staining.

lymphovascular invasion, tumor grade and CHD5 expression levels. Subset analyses were performed for patients receiving only adjuvant therapy of any kind and patients receiving only adjuvant gemcitabine based chemotherapy. All analyses were conducted using the Statistical Package for the Social Sciences 20.0 for Windows (SPSS Inc., Chicago, IL, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DISCLAIMER

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